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DIPARTIMENTO DI SCIENZE DEL FARMACO

SCUOLA DI DOTTORATO DI RICERCA IN
BIOLOGIA E MEDICINA DELLA RIGENERAZIONE
INDIRIZZO INGEGNERIA DEI TESSUTI E DEI TRAPIANTI
XXV CICLO

TESI DI DOTTORATO

ACELLULAR MATRICES AS TOOL FOR RENAL PROGENITOR DIFFERENTIATION STUDIES AND TISSUE ENGINEERING OF BLOOD VESSELS

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RIASSUNTO

Le matrici acellulari rappresentano uno *scaffold* promettente per l'ingegneria tessutale. Infatti, la matrice extracellulare costituisce un supporto sito-specifico che favorisce la crescita e il differenziamento delle cellule di qualsiasi organo.
Ad oggi, le tecniche dell'ingegneria tessutale sono utilizzate sia per lo sviluppo *ex vivo* di sostituti tessutali, che per studiare la proliferazione e la differenziazione delle cellule quando si trovano all'interno di uno *scaffold* tridimensionale.

In questo lavoro di tesi, i due seguenti progetti sono andati a valutare entrambe le potenzialità di matrici acellulari tessuto-specifiche:

1- valutazione della capacità della matrice acellulare di indurre il differenziamento di progenitori renali da fluido amniotico in cellule renali mature;

2- valutazione della matrice acellulare per la sostituzione di vasi sanguigni.

1- La matrice acellulare renale è stata utilizzata per valutare la capacità differenziativa di progenitori renali da fluido amniotico in modo da valutarne una futura applicazione terapeutica. I progenitori renali sono stati seminati sulla matrice acellulare renale, che, *in vitro,* ne ha promosso la proliferazione, il mantenimento del fenotipo podocitario e la differenziazione in cellule tubulari. Per valutare i*n vivo* il potenziale differenziativo di queste cellule, la matrice da sola o ripopolata con le cellule è stata impiantata all'interno di un rene di topo nudo. I progenitori renali si sono ulteriormente differenziati, si sono integrati all'interno delle strutture tubulari dell'ospite e hanno promosso la migrazione di cellule differenziate murine all'interno dello *scaffold*.

2- La matrice acellulare di aorta è stata utilizzata per lo sviluppo di sostituti vasali. Nonostante vasi autologhi o costituiti di polimeri sintetici vengano già utilizzati nella pratica clinica per la ricostruzione di vasi di piccolo diametro (<5 mm), numerosi sono gli svantaggi legati al loro utilizzo, quali l'iperplasia della tonaca intima e la degenerazione arteriosclerotica. Lo scopo di questo studio è stato quello di sviluppare sostituti vasali utilizzando come *scaffold*

vasi decellularizzati. Matrici acellulari da sole o ripopolate con cellule endoteliali da microcircolo sono state impiantate nell'aorta di ratto Lewis. Come osservato negli impianti di sola matrice acellulare, la mancanza della copertura endoteliale portava all'iperplasia dell'intima e all'aumento di incidenza dei processi trombotici, sottolineando la necessità di reendotelizzare *in vitro* il vaso decellularizzato prima dell'impianto *in vivo*. Infatti, i sostituti vasali costituiti da matrice acellulare e cellule endoteliali da microcircolo hanno dimostrato di avere una buona resistenza al flusso e non presentavano trombi al loro interno. Sebbene questi vasi fossero assottigliati e mostrassero una leggera iperplasia della tonaca intima, questo approccio presentava due principali vantaggi: permetteva di ottenere sostituti vasali in un tempo clinicamente utile ed eliminava la necessità di rimuovere vasi sani per ottenere cellule endoteliali autologhe.

ABSTRACT

Acellular matrices (AMs) seem to be a very promising scaffold in Tissue Engineering (TE) and can be considered as temporary inductive site-appropriate templates to support the growth, differentiation, and function of the parenchymal cell population of each organ. Nowadays, TE techniques are used both to develop tissue substitutes *ex vivo* and as reliable tool to investigate cell behaviour, differentiation and proliferation in 3-dimentional environments.

In this work the following two different projects have investigated both potentialities using tissue-specific AMs:

1- influence of AMs on differentiation of kidney progenitor cells from amniotic fluid into mature renal cells;

2- AMs as biomaterial to develop vessel substitutes.

1- Kidney AMs (KAMs) were used to evaluate the differentiation of kidney progenitor cells from amniotic fluid into mature renal cells in order to better understand whether they could be suitable for future application in therapy. Renal progenitors were seeded into KAMs, which led them to proliferate, maintain podocyte phenotype and differentiate into tubular cells *in vitro*. To further evaluate the differentiative potential of KAMs, grafts composed of KAM with or without cells were intrarenal implanted into nude mice. *In vivo*, progenitors from amniotic fluid expressed mature renal markers, attracted inside KAMs differentiated murine cells and integrated into host structures.

2- Although autologous vascular grafts and artificial materials have been used for reconstruction of small diameter (<5 mm) blood vessels, the poor availability of vessels and the occurrence of intimal hyperplasia and progressive atherosclerotic degeneration represent shortcoming of these vascular prostheses. Therefore, this study aimed to develop AM-based vascular grafts. Both aorta AMs (AAMs) alone and AAMs previously reendothelized with skin microvascular endothelial cells (ECs) were *in vivo* implanted and analyzed. The lack of reendothelization, leading to intimal hyperplasia and increased incidence of thrombosis observed in AAMs grafts,

have indicated the need to provide *in vitro* an endothelial coverage of decellularized tissue. Indeed, grafts composed of AAM and skin microvasculature ECs shown good patency and no thrombi. Although these grafts appeared narrowed and a moderate hyperplasia has been detected in the inner layer, they presented two main advantages: they were obtained into a clinically relevant time frame and eliminated the need to remove healthy vessels for collecting autologous ECs.

INTRODUCTION

1. TISSUE ENGINEERING

End-stage disease is a serious, growing and costly issue. At the present, definitive treatment for end-stage organ failure is allogenic transplantation. However, a combination of unremitting demands, expensive and potentially dangerous immunosuppression, and the requirement that donor organs be physiologically viable means that the clinical need will never be met and many patients on transplantation waiting list will die before a donor organ becomes available. Patients fortunate enough to receive a donor organ endure life-long immunosuppressive therapies with its associated morbidity and are at risk of acute or chronic organ rejection (Badylak *et al,* 2012).

Langer and Vacanti postulated that living organs might be designed and built based on the principle of biological science and technologic advances in the engineering disciplines (Langer *et al*, 1993). This new field, named Tissue Engineering (TE), combines aspects of cell biology and transplantation, material science and biomedical engineering to develop biological substitutes that can restore and maintain the function of damaged tissue and organs.

TE strategy could be used also as a reliable tool to investigate cell behaviour, differentiation and proliferation pathways in 3-dimentional environments, in order to decrease *in vivo* experiments. Besides, scaffolds miming native supports could be used *in vitro* as an alternative approach to improve cells proliferation and differentiation.

The two basic components of this strategy are cells and biomaterials. The introduction of cells is designed to stimulate regeneration, promote vascularisation and supplement the production of hormones and growth factors. Biomaterials, natural or synthetic scaffolds, guide the direction of new tissue growth, provide the proper spatial environment to restore tissue structure and function and may introduce bioactive molecules, attracting cells and growth factors from the host after implantation (Atala, 2012).

1.1 Cell types used in TE

Depending on the organ to be restored, several cell types are currently investigated in order to obtain a tissue that can perform the appropriate physiologic/metabolic

duties. Briefly, cells used in TE can be tissue-specific differentiated cells or stem cells.

1.1.1 Tissue-specific differentiated cells

These cells are harvested from the specific organ to be regenerated, cultured *ex vivo* and used in the same patient without rejection, in an autologous manner. One of the limitation of this technique has been the inherent difficulty of growing specific cell types in large quantities.

1.1.2 Stem cells

The interest about stem cells has been increasing over the past years, since their discovery in the early '90s. Stem cells might be a promising tool for regenerative purposes because of their capability to become almost any cell of an adult organism. It is universally accepted that a stem cell possesses two fundamental characteristics: long term self-renewal and pluripotentiality.

Self-renewal describes the unique capability of these cells both to reproduce itself indefinitely and to produce cell progeny that matures into more specialized, organ-specific cells.

Pluripotentiality is defined as the ability of a stem cell to give rise to different tissues. Based on their capability to differentiate into different cell types, stem cells are divided into different categories. Pluripotent stem cells are defined by the ability to differentiate, under certain stimuli from the surrounding environment, into cells of all the three germ layers and germ cells. A cell is defined as multipotent if can give rise to more than one cell type and unipotent if it can differentiate into one cell type.

Embryonic Stem Cells

Embryonic Stem Cells (ESCs) are collected from the Inner Cell Mass (ICM) of the blastocyst at five days from the fertilization of the egg. The blastocyst includes three structures: the trophoblast, which is the cell layer surrounding the blastocyst; the blastocoel, which is the hollow cavity inside the blastocyst; and the ICM, which is a group of approximately 30 cells at one end of the blastocoel. ESCs are defined as pluripotent. For this reason, ESCs have been widely investigated for their wide capability to differentiate into any cell line of the body, being a reliable tool for cell development and differentiation pathway studies. However, their clinical

application is limited by ethical issues, their origin that can evoke an immune response, and the potential to form teratomas.

Fetal Stem Cells

Fetal Stem Cells can be isolated from two different sources, the fetus proper and the supportive extra-embryonic structures (amniotic fluid, Wharton's jelly, placenta and amnion).

Several population have been obtained from extra-embryonic structures; fetal cells grow well in culture, are able to differentiate into multiple cell types and may be less likely to be rejected following transplantation. Besides, the extracorporeal nature of fetal stem cell source facilitates isolation, increases the number of stem cells that can be obtained and overcomes ethical concerns (Marcus *et al*, 2008).

In particular, De Coppi *et al* (2007) have shown that amniotic fluid contains a novel type of stem cell which is capable of being maintained in an undifferentiated state in culture for long periods and can be induced to differentiate into many different cell types. Their pluripotency, high proliferation rates, multi-differentiation capability and lack of teratoma formation when injected *in vivo* make them attractive candidates for cell sourcing. In addition, there are no serious ethical issues with the use of these cells, which is an advantage over other stem cells such as ESCs and induced Pluripotent Stem cells (iPS). Very recent exciting results using amniotic fluid stem (AFS) cells or AFS-combined engineered tissues for therapeutic applications have encouraged their use in the field of regenerative medicine in more advanced and broader manners (Joo *et al*, 2011).

Adult Stem Cells

Adult Stem Cells (ASCs) are located within the tissues of the adult body. Under specific stimulation, they undergo differentiation and replace the loss of cells in an injured compartment. A specific organ localization, called niche, is thought to harbor the stem cells in an environment that protects cells from differentiation.

Examples of ASCs are hematopoietic stem cells (HSCs). HSCs, localized within the bone marrow, are the most important adult stem cell line. HSCs are commonly used for the treatment of leukemia diseases. Furthermore, HSCs were shown to differentiate into myocites, endothelial cells, hepatocytes and epithelial cells of liver, gut, lung and skin.

In bone marrow another population, called bone marrow stromal stem cells (BM-MSCs) shows to differentiate into mesenchymal lineages. BM-MSCs were shown capable to differentiate *in vitro* into adipocytes, chondrocytes, muscle cells, tendons, osteoblasts and endothelial cells. *In vivo* experiments reported successful differentiation of mouse BM-MSCs into brain astrocytes, glial cells, CNS cells, hepatocytes, endothelial and myocardial cells in adult mice (Raff, 2003).

Somatic Cell Nuclear Transfer and Induced Pluripotent Stem Cells

Scientific knowledge allows us to modify cell genetic background and gene expression and to obtain different types of exogenous stem cells. Somatic Cell Nuclear Transfer (SCNT) cells were derived from the injection of an endogenous somatic cell nucleus within an oocyte. The result was the creation of a pluripotent cell, capable of being implanted in utero or used to retrieve new stem cell lineages.

iPS cells were obtained with insertion of pluripotent genes within the DNA of a somatic cell. Retroviral introduction of transcription factors OCT-4, SOX-2, KLF4 and MYC induced pluripotency within somatic cells. Recent studies have shown that OCT-4 and SOX-2 could be combined with other genes to produce iPS cells. iPS cells were able to participate to the embryonic development when injected in a blastocyst.

However, the efficiency of the reprogramming process is jeopardized by the low yield of transfection and the long time of reprogram. Moreover, the employment of viral delivery system like lentiviral vectors might increase the risk of insertions of foreign genetic sequences in host genome thus causing gene mutations and cell transformation. Recently, small molecules able to modulate specific targets in receptor signaling and epigenetic machinery have been used to improve the reprogramming process and/or replace some transcriptional factors, thus partially or totally avoiding the host genome involvement. In this context, histone deacetylase inhibitors (HDACIs), such as valproic acid (VPA), thricostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA), induce the hyperacetylation of histones modifying chromatin moiety and affecting gene expression (Huangfu D *et al*, 2008).

1.2 Biomaterials used in TE

The biomaterial itself should be able to i) naturally provide cell attachment and support; ii) dispose of sufficient area to allow cell proliferation; iii) develop the

ability of shaping specific structures; iv) *in vivo* degrade without releasing toxic materials; iv) allow tissue remodelling and resorption avoiding foreign body reaction and iv) allow ingrowth of host cells. Furthermore, it can also provide mechanical support against *in vivo* forces such that the predefined 3-dimensional structure is maintained during tissue development. Two classes of biomaterials have been used for engineering tissues and organs: synthetic polymers and natural biomaterials.

Synthetic biomaterials

Synthetic biomaterials present the following advantages in comparison with natural scaffolds: tightly control of physical properties, such as mechanical strength, degradation rate and pore size, and production with fewer batch-to-batch variations. One drawback of the synthetic polymers is lack of biologic recognition, although a number of groups are attempting to design synthetic scaffolds, which incorporate proteins or other molecules to assist in recognition (Atala, 2012). So, adhesion molecules can be adsorbed or covalently bound to the surface of scaffold. Extracellular matrix (ECM) adhesion proteins, such as fibronectin, collagen and laminin, present some disadvantages in the view of medical applications. They can elicit immune response, since they are isolated from other organisms and need to be purified. They also need to be refreshed continuously, because they are object of proteolytic degradation. On the contrary, small peptides, containing only the sequence responsible for cell adhesion, are characterized by higher stability, easier characterization, and possibility to be packed with a higher density on surfaces. Thus, their use can overcome most of problems connected to ECM proteins. For example, small peptides can be designed to contain RGD sequence (Arg-Gly-Asp) which mediates cell-adhesion via cell membrane integrin receptors, or heparin binding sequences able to interact with cell membrane heparin sulphate proteoglycans.

The major classes of synthetic biomaterials include glycolic acid derivatives (PGA), lactic acid derivatives (PLA), and other polyester derivatives (as poly(lactic-co-glycolic acid, PLGA). These polymers have gained Food and Drug Administration approval for human use in a variety of applications, including sutures. The degradation products of PGA, PLA, and PLGA are nontoxic, natural metabolites that are eventually eliminated from the body in the form of carbon dioxide and water.

Because these polymers are thermoplastics, they can easily be formed into a 3-dimensional scaffold with a desired microstructure, gross shape, and dimension by various techniques. Electrospinning has been used to quickly create highly porous scaffolds in various conformations.

Natural biomaterials

Natural scaffolds are made up of protein or carbohydrates with particular biochemical, mechanical, and structural properties. They can be derived from plant or animal sources, and are mostly found to be both biocompatible and biodegradable. These scaffolds have an increased advantage due to the presence of multifunctional groups on scaffold surfaces, which can be tailored according to specific applications. Examples of natural biomaterials are collagen, chitosan, hyaluronic acid, fibrin, and gelatin, which have been applied for the repair and reconstruction of several tissues. Even though natural scaffolds have been applied for a variety of tissue-engineering applications. Limitations include the inability to control or modify the chemical and biologic properties of these scaffolds for specific applications (Patel *et al*, 2008).

Among natural biomaterials, acellular tissue matrices (AMs) have been successfully used both in pre-clinical animal studies and in human clinical applications. AMs are prepared by removing cellular components from tissues and are commonly used to facilitate the constructive remodeling of a variety of tissues.

The preparation of a 3-dimensional, AM scaffold from an intact mammalian organ requires several processing steps, each of which can markedly affect the structure and composition of the biomaterial and the associated host response that these scaffolds will elicit when utilized as templates for organ reconstruction. The effective removal of antigenic epitopes associated with cell membranes and intracellular components of organs and tissues is necessary to avoid, or at least minimize, adverse immune responses by allogeneic or xenogeneic recipients. The decellularization process that must retain the native composition and structure of the associated matrix, typically involves exposure of the tissue to detergents, proteases, and chemicals by perfusion of the native vasculature. Commonly used protocols include the perfusion of chemical or enzymatic agents and physical methods, such as sonication, freezing, and thawing, coupled to shaking in order to disrupt cell membranes, release cell contents, and facilitate the rinsing and removal

of cell remnants from the ECM. Combinations of these various approaches are typically used to maximize the efficiency of the process for each tissue and organ. Organ decellularization may involve the delivery of chemical agents by vascular perfusion. As a general rule, the use of detergents and chaotropic agents such as TRITON X, sodium dodecyl sulfate, and sodium deoxycholate should be minimized whenever possible to avoid damage to the ultrastructure and composition of the native ECM.

The specific composition varies depending on the source tissue/organ from which the AM is prepared. ECM proteins are arranged in a unique, tissue-specific, 3-dimensional ultrastructure and are ideally suited to the tissue or organ from which the AM is harvested. The peculiar structure and composition can be largely preserved by the appropriate use of processing steps required for the decellularization of the tissue. In general, however, AMs from all organs consists mainly of type I collagen, glycosaminoglycans, fibronectin, laminin, and various types of growth factors. Furthermore, AMs maintain organ-specific structures, such as the collagen type IV and laminin-rich basement membrane of blood vessels. Ultrastructural characteristics of the matrix appear to play important roles in modulating the cell behaviour either by regulating cell ability to migrate into and attach to specific locations within the scaffold or by influencing tissue-specific phenotypic differentiation. Cell proliferation, migration, and differentiation, as well as processes such as angiogenesis are mainly regulated in part by cell-signaling mechanisms involving soluble molecules. Thus, the biologic signaling activities provided by degradation products of AMs have a marked effect on the host-remodeling response following *in vivo* implantation. These scaffold materials can be considered as temporary inductive site-appropriate templates to support the growth, differentiation, and function of the parenchymal cell population of each organ (Badylak *et al*, 2011).

2. TISSUE ENGINEERING IN KIDNEY DISEASE

2.1 End Stage Renal Disease and Chronic Kidney Disease

End Stage Renal Disease (ESRD) is a condition of chronic and progressive injury of the kidney, leading to a complete failure of the renal system. ESRD usually occurs when renal functionality is less than 10% of normal activity (Perin *et al*, 2008). According to the 2007 United States Renal Data System, the number of United States patients in treatment for ESRD was 400,000, with more than 20,000 waiting for organ transplantation. Predictions for the year 2020 are showing an increase in patients undergoing dialysis and in need of kidney replacement. Progression to ESRD can be simplified within two major processes known as Acute Kidney Failure (AKF) and Chronic Kidney Disease (CKD).

AKF is characterized by sudden and fast kidney function deterioration. Pathological kidney functionality is characterized by a decrease in filtration rate, starting from previously called pre-renal acute kidney injury and up to unresponsiveness. Kidney stones, infections, cancer or drug intoxication can be causes for AKF (Lameirea *et al*, 2008). Common treatments to improve renal perfusion increase cardiac output, replenish the circulating volume, enhance cardiac inotropy, and induce vasoconstriction. About 4% of all critically ill patients with AKF will require dialysis.

CKD is recognized as a major health problem affecting approximately 13% of the United States population. Numbers of prevalent CKD patients will continue to rise, reflecting the growing elderly population. CKD is defined as the presence of kidney damage, manifested by abnormal albumin excretion or decreased kidney function that persists for more than 3 months. Typically, kidney function is quantified by glomerular filtration rate (GFR), the rate at which an ultrafiltrate of plasma is produced by glomeruli per unit of time, and is the best estimate of the number of functioning nephrons or functional renal mass. The early stages of CKD (GFR between 90 and 60 mL/min per 1.73 m^2) are manifested by kidney damage and are generally asymptomatic: the kidney functions normally but the risk for progressive disease is significant. As kidney disease worsens, renal function begins to deteriorate (GFR between 29 and 15 mL/min per 1.73 m^2). Eventually, kidney failure (GFR < 15 mL/min per 1.73 m^2) occurs and kidney replacement therapy is required (NKF-KDOQI, 2007). Common origins for CKD are pathologies affecting the kidney compartment like analgesic nephropathy, glomerulonephritis, kidney stones,

obstructive uropathy and reflux nephropathy, lupus, and polycystic kidney disease, genetic malformations or diseases affecting other organs, like diabetes and hypertension. Complications derived from CKD are various. The loss of function is usually coupled with an increase of fibrosis, amyloid deposition and glomeruli destruction. Major sequelae of CKD include continued progression of the disease and development of kidney failure requiring kidney replacement therapy, development and/or progression of cardiovascular disease, anemia, and bone disease.

There are several therapeutic and pharmacological tools used by clinicians to slow the progression and symptoms of ESRD, but the only effective treatments up to now are dialysis and transplantation.

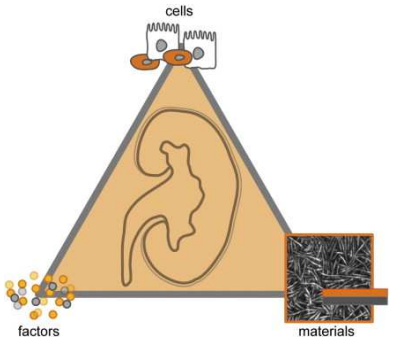
Currently, in the early stages, CKD drug therapy is limited to administration of antihypertensive agents to decrease blood pressure, and consequentially decrease the risk of injury provoked by high blood pressure, and limit proteinuria. Although, pharmacological treatments are a good start point to treat kidney disease, mechanisms leading to ESRD are multiple and very complex and the administration of one or more drug is not enough to treat and cure the CKD. Even if on pharmacological therapy many patients eventually require renal replacement therapy, or dialysis. Dialysis is a clinical procedure that substitutes the loss function of the kidney for what concerns the blood purification. Dialysis is not a complete replacement therapy because it provides only blood filtration while kidney is an endocrine organ responsible for the secretion of hormones that are critical in maintaining hemodynamics (renin, angiotension II, prostaglandins, nitric oxide, endothelin, and bradykinin), red blood cell production (erythropoietin), and bone metabolism (1,25-dihydroxyvitamin D3 or calcitriol). In addition, although life-sustaining, dialysis does not provide a good quality of life and several side effects may occur such as hypotension, arrhythmia, and complications of vascular access placement.

Kidney transplantation from a live donor is the first choice for eligible patients who require renal replacement therapy: it has lifestyle advantages and is cheaper than dialysis. Nonetheless, availability of donor kidneys is very limited. Many adults on the deceased donor waiting list will die on dialysis before they receive an organ. Thanks to the use of immunosuppressive drugs, by now it is possible to transplant not well-matched organs. However, immunosuppression takes its toll in both the

short and long term. In the short term, infection is a particular concern, especially with viruses such as cytomegalovirus, while, in the long term, the incidence of most cancers is increased in patients who are immunosuppressed.

2.2 Renal regenerative medicine

In order to overcome the limits of current therapies, scientists and clinicians have looked for alternative approaches for CKD management. In the last years TE has grown as an alternative for several diseases and the possibility of restoration of kidney tissue using cells, regenerative factors, biomaterials, or combination of these three, is approaching (Dankers *et al,* 2011).



Renal regenerative medicine. Three possible strategies, or combination of them that can be applied to regenerate and/or engineer renal tissue (Dankers *et al*, 2011)

2.2.1 Cell-based approaches

Many investigators have searched for small subpopulation of resident stem cells that might be responsible for the rapid proliferative response after renal injury while in healthy kidney they stay in a dormant state. In rat kidneys renal stem cells were discovered among tubular epithelial cells and in the papilla and they have been shown to differentiate into renal tubules when injected under the renal capsule. However, the clinical significance of these observations is still unclear (Gupta *et al*, 2006).

BM-MSCs seem to contribute to renal regeneration. After damage, they can replace tubular, mesangial, interstitial, endothelial cells and podocytes, but the relative contribution is very low and does not exceed a few percent of the total proliferating cell fraction. However, they can produce a variety of paracrine factors, such as VEGF (Vascular Endothelial Growth Factor), IGF (Insulin-like Growth Factor), bFGF (Fibroblast Growth Factor), HGF (Hepatocyte Growth Factor) and TGFβ

(Transforming Growth Factor β) that can enhance repair. Despite the optimism about their use for renal regeneration, they have been shown to be ineffectiveness in case of kidney fibrosis and to differentiate towards myofibroblasts producing excessive collagens.

A number of studies have explored the possibility to generate renal epithelia from ESCs. After injection, ESCs have been shown to integrate into the tubuli of developing kidneys. However, differentiation towards a specific cell lineage is very complicated; it depends on factors secreted in the microenvironment and it is difficult to mimic *in vitro* (Dankers *et al,* 2011). Besides, there are still several ethical concerns about their clinical use.

Perin *et al* (2010) have shown that amniotic fluid could represent a novel source of stem cells that may function to modulate the kidney immune milieu in AKF. When injected into the damaged kidney, amniotic fluid stem cells provided a protective effect, ameliorating AKF in the acute injury phase by decreasing the number of damaged tubules and apoptosis therein and by promoting proliferation of tubular epithelial cells.

Recently, Sedrakyan *et al* (2012) demonstrated that stem cells from amniotic fluid could be beneficial also in kidney diseases characterized by progressive renal fibrosis. Amniotic fluid stem cells did not differentiate into podocyte-like cells, but the mechanism of renal protection was probably the paracrine/endocrine modulation of both pro-fibrotic cytokine expression and recruitment of macrophages to the interstitial space.

Da Sacco *et al* (2010) demonstrated that human amniotic fluid provides a new source for renal progenitor cells. These cells were grown in culture for several passages and expressed typical renal markers. The identification of specific renal progenitors suggests that human amniotic fluid may represent a valuable new source of cells for regenerative therapies that may be applicable to a broad range of renal disease.

2.2.2 Growth factor-based approaches

Exogenous growth factor administration to enhance renal regeneration has been studied in large detail. The up-regulation of growth factor genes promotes renal repair processes. Systemic injection of EGF and HGF successfully enhanced recovery and survival after acute kidney injury in animal models (Hammerman *et*

al, 1994). However, a major disadvantage is that these therapies might lead to unwanted side effects in other organs, especially when the drug is administered systemically.

2.2.3 Biomaterial-based approaches

In the future, a possible solution for patient with CKD, besides dialysis or transplantation, might be a tissue-engineered kidney, which should be able to replace all kidney functions, including endocrine and metabolic activities.

A new approach is the bioengineering of an extracorporal renal device using a membrane and a single renal cell type that has to form a monolayer of cells in order to replace critical endocrine and metabolic renal function. Such a bioartificial kidney might be applied as a renal assist device and exert its function when placed in series with a conventional hemodialysis module (Aebischer *et al* ,1987).

Humes *et al* (1999) designed a membrane composed of human epithelial cells in a hollow fiber cartridge that was characterized both by pore selectivity and hydraulic permeability as the native kidney. The transport of electrolytes was absent but the activity of renal epithelial cells could attenuate the consequences of septic shock by modulating plasma cytokine levels.

Few experiments have been conducted where renal cells were cultured *in vitro*, seeded into a polyglycolic acid polymer scaffold and subsequently implanted into athimic mice. Over time, formation of nephron-like structures was observed within the polymer (Amiel *et al*, 2000). These preliminary results, when improved, could easily be used to produce 3-dimensional functional renal structures that can be used in *ex-vivo* or *in vivo* filtering units. Yoo *et al* (1996) harvested mouse renal cells and seeded them onto a polycarbonate tube. This device was connected at one end to a silastic catheter which terminated into a reservoir, and then implanted subcutaneously in athymic mice. Histological examination demonstrated extensive vascularization as well as formation of glomeruli and highly organized tubule-like structures. Nonetheless, since adult cells are completely differentiated and their response to growth factors can be absent or different from cells in the developing kidney, the seeding and the integration of these systems may fail or be partial.

Therefore, whole-organ approaches, such as reseeding of AMs with stem cells have been investigated. Ross and colleagues (2009) infused mouse ESCs through the renal artery and the ureter into an isolated rat renal AM and showed proliferation

and cell-specific differentiation of the stem cells within the glomerular, vascular and tubular compartments. In an other study acellular scaffolds with maintained expression patterns were reseeded with cells obtained from donor kidneys of non-human primate and fetal kidney cells. AMs demonstrated the capacity to support cells attachment and migration, promoting cell repopulation (Nakayama *et al*, 2010). At the present, much work needs to be done for *ex-vivo* kidney regeneration with AMs and cell therapy approaches to become a viable clinical option.

3. TISSUE ENGINEERING IN VASCULAR DISEASE

3.1 Vascular disease

Vascular disease can be divided into Coronary (CAD) and Peripheral Artery Disease (PAD).

CAD, characterized by anatomic or functional alterations of coronary artery, can be congenital, due to a birth malformation and rather rare, or acquired, as result of a narrowing of the blood vessels. It is the leading cause of death worldwide.

PAD refers to the obstruction of arteries that carry blood to the legs, arms, stomach or kidneys. There are two types of PAD:

- functional PAD doesn't involve defects in the structure of blood vessels that are not physically damaged. This disease has often symptoms related to “spasm” that may come and go and it can be caused by temperature, emotional stress or smoking.
- organic PAD is caused by structural changes in the blood vessels. Examples could include inflammation and tissue damage. It can be caused by fatty buildups (atherosclerosis) in the inner wall of arteries which block normal blood flow.

3.2 Vascular disease therapy and vascular regeneration

PAD management includes lifestyle changes, medicines or both. Currently pharmacological therapy is limited to the administration of antiplatelet agents and cholesterol-lowering agents (statins). When the drug therapy is not enough, angioplasty or bypass surgery may be needed.

Coronary and peripheral artery bypass grafting is commonly used to relieve the symptoms of vascular disease. The grafts usually consist of either mammary artery or saphenous vein harvested from the patient. However, the supply of native vessels may not be sufficient for multiple bypass or repeat procedures. Furthermore, saphenous vein in the elderly patients is prone to thrombi, neointimal formation,

atherosclerosis or aneurysm when transplanted into high-pressure arterial sites. Thus, the use of materials other than arterial or venous conduits is required. Synthetic vascular prostheses, such as Dacron fabric grafts and expanded polytetrafluoroethylene (ePTFE), have been developed to overcome the limited supply of native graft materials. They perform reasonably satisfactorily in high-flow, low-resistance conditions such as the large peripheral arteries, but they are not as suitable for small calibre arterial reconstructions (e.g. coronary or lower leg circulation). Indeed, they are prone to thrombus induction, embolism and occlusion, may harbour bacteria (resulting in graft infection) and may act as a foreign body in the patient. They are also associated with poor healing, lack of compliance and excessive intimal hyperplasia, particularly near the sites of anastomosis (Thomas *et al*, 2003). Overall, synthetic grafts are not suitable for reconstruction of small-diameter (internal diameter <5 mm) arteries, due to thrombosis, limited reendothelialization, and neointimal hyperplasia, owing mainly to the inherent properties of the synthetic materials.

To avoid the synthetic scaffold limits, some groups have continued to use synthetic graft materials as a base for further coats of active materials, such as collagen, fibrin, gelatin, Growth Factor Stimulating Factor (GSF) or RGD. Synthetic grafts have also been used as a scaffold for supporting cells. However, vascular cells cannot remodel ePTFE or Dacron in the same way as they remodel normal ECM components such as collagen and elastin and they will detach when exposed to blood flow. An other approach is to passivate synthetic grafts with ‘‘biologicals’’ including heparin, hirudin, prostaglandins, growth factors, anticoagulant peptide sequences, dextran derivatives (to prevent coagulatory events), and antibiotics (to minimise graft infections). These molecules are designed to either remain *in situ* (and thus act locally) or to be released into general circulation (Thomas *et al*, 2003).

To overcome the limits of current therapies, scientists and clinicians have looked for alternative approaches using new biomaterials and cells in order to obtain small diameter vessel substitutes.

3.3 Biomaterials used in vascular regeneration

For a successful regenerated vascular construct the scaffold material plays a major role in all the tissue engineering strategies as it provides the basic framework for cell growth. Various materials have been utilized for blood vessel engineering,

including synthetic biodegradable polymers and natural biomaterials. Most of these scaffolds have also undergone preclinical studies in animals.

3.3.1 Synthetic biomaterial

Synthetic scaffold are easily available and inexpensive. These polymers can be precisely modified to adjust their degradation rate, biocompatibility and elasticity. The most preferred and widely used materials for bioresorbable grafts included polyglycolic acid (PGA), polydioxanone (PDO), and polylactide (PLLA) which are FDA approved.

PGA is the most commonly studied scaffold. Its highly porous structure allows nutrient diffusion and subsequent neovascularisation. Moreover, it is easily handled and fabricated into different shapes. As an approach to develop small-diameter vascular conduits, Niklason *et al* (1999) seeded bovine aortic smooth muscle cells (SMCs) on tubular PGA scaffolds and cultured for 8 weeks under conditions of pulsatile pressure before seeding with endothelial cells (ECs). They found that the endothelialisation was present only in pockets of the graft surface with persistent PGA remnants. This failure of endothelialisation may be due to cytotoxic degradation products of PGA or lack of cell signalling support.

Poly ε-caprolactone (PCL) is another popular biomaterial with a slow degradation profile, and it is eliminated through macrophage and giant cell encapsulation. Therefore, it is most suitable for the design of long-term implantable systems.

The first clinical application of an artificial vessel based on biodegradable scaffold was reported by Shin'oka *et al* (2001) in a 4-year old girl. A Polycaprolactone–polylactic acid copolymer tube was reinforced with woven PGA and then seeded with the patients own venous ECs. After that, several patients underwent tissue engineered graft implantation with cultured autologous venous cells. There was no evidence of aneurysm formation, graft rupture, graft infection, or ectopic calcification. Similar results were obtained using BM-MSCs (Shin'oka *et al,* 2005).

Although synthetic materials are advantageous over the natural polymers in terms of strength and tunable degradation characteristics, they also lack the appropriate cell signaling cues which mainly limit the cell maturation, differentiation and appropriate ECM secretion necessary for tissue regeneration. These materials may also lead to cell delamination from the surface which is also a critical issue when it comes to luminal endothelialisation and blood shear flow characteristics.

Modification of synthetic surfaces by attaching specific cell responsive groups, like RGD peptide sequences or heparinized surfaces, is being explored to overcome these issues (Thomas *et al*, 2003).

3.3.2 Natural biomaterials

An important alternative to synthetic materials is the use of AMs derived from *ex vivo* tissues. Decellularized biological scaffolds have the advantage of being rich in the cell signalling components essential for cell adhesion, migration, proliferation and differentiation. Preserved AM components include collagen, elastin and glycosaminoglycans. Intact collagen and elastin fibers ensure retention of tensile strength and elastic recoil properties, and there is evidence to suggest that they inhibit SMC proliferation, an early step of intimal hyperplasia. Glycosaminoglycans, which include chondroitin, dermatan, heparan and heparin sulphate, play a crucial role in EC adhesion and proliferation, inhibition of SMC proliferation and migration following injury or inflammation, in addition to their antithrombotic properties. Biological vascular substitutes also display a greater resistance to infection compared to synthetic grafts (Yow *et al*, 2006).

One notable example of a decellularized tissue that is being used in vascular tissue engineering is the small intestinal submucosa (SIS) that is seen to retain angiogenic growth factors, such as b-FGF and VEGF. At first, decellularized SIS without any modification was used as vascular substitute showing a good patency rate as a large-diameter graft (Lawler *et al*, 1971). After almost 30 years, Huynh and colleagues (1999) constructed a scaffold from a collagen-based biomaterial derived from SIS and bovine collagen type I. The inner lining was treated with heparin and this acellular graft was implanted into rabbit aortas demonstrating promising results as small-diameter graft.

Various other decellularized matrices were also used like bovine ureters, porcine carotid artery and aorta and even canine carotid arteries (Clarke *et al*, 2001; Conklin *et al*, 2002). Decellularized human umbilical arteries were also implanted into nude rats as abdominal aorta interposition grafts which remained mechanically intact and patent for up to 8 weeks (Gui *et al*, 2006).

However, the luminal surface of these decellularized matrices without EC lining carries a substantial risk for thrombosis when exposed directly to the blood flow. Hence in recent years, much work has been focused on recreating tissue-engineered

vascular grafts by recellularizing biomaterials with host vascular cells prior to implantation. Bader *et al* (2000) used decellularized porcine aortas, which were then repopulated with human myofibroblasts and ECs derived from saphenous vein. After 2-3 weeks, the graft was fully repopulated and immunologically acceptable to the host.

Although non-human decellularized vessels are very easy to access and promote site-specific remodeling and regeneration by the host, they face chances of transmission of animal pathogens to human being when considering the clinical scenario.

Alternative vascular substitutes are based on collagen, fibrin or gelatin. Collagen-based conduit revealed to be fragile and they need a wrapping with a reinforcing synthetic polymer. However, the reinforcing materials on account of being non-degradable were seen to adversely affect the remodeling response which led to loss of compliance in post-implantation period (Matsuda *et al*, 1995).

Swartz *et al* (2005) engineered implantable small-diameter blood vessels based on ovine SMCs and ECs embedded in fibrin. These grafts, implanted in the jugular veins of lambs, were well integrated into the native vessel and demonstrated patency up to 15 weeks, similar blood flow rates and matrix remodeling as the native vessels.

Another breakthrough in the fabrication of tissue-engineered grafts was the cell sheet approach developed by L'Heureux and colleagues (1998) where an innovative graft was developed exclusively from cultured human cells. Sheets of SMCs and fibroblasts were grown to over-confluence and then assembled over a mandrel to form a tubular structure that was subsequently cultured for 6–8 weeks. During this incubation period, the autologous cells arranged themselves circumferentially and produced large amounts of ECM, thus establishing a structural integrity capable of withstanding pressures in excess of 2000 mmHg. The inner lumen of the construct was then seeded with ECs to promote non-thrombogenicity. Recently, this technology has been translated to clinical use as arteriovenous fistula shunts in hemodialysis patients with promising results. Although the robustness and non immunogenic nature of self-assembly grafts hold great promise, the primary limitation is the amount of time required for culturing the cells. The extended time frame raises cost and impairs the 'off-the-shelf' capability of such a technology (L'Heureux *et al*, 2007; McAllister *et al*, 2009).

3.4 Cells used in vascular regeneration

The combination of natural or synthetic scaffold together with vascular cells is considered the most promising option. The endothelium is not only a smooth inert surface that facilitates laminar blood flow through the blood vessel, but also a dynamic organ with an active role in coagulation homeostasis, the sensing and transduction of the haemodynamic forces of circulation, and the cell metabolism of the vascular wall. For these reasons, surface endothelialization has a great role in intermediate the long-term patency and it is crucial to prevent intimal hyperplasia and graft occlusion. One aspect of endothelial protection is the physical barrier it forms to prevent contact with subendothelial components of the arterial wall and activation of the coagulation cascade. The ideal cell source should be non immunogenic, functional and easy to isolate and expand in culture.

The non immunogenic autologous ECs and SMCs isolated from patients themselves represent the first choice of cells for tissue engineering. Reports show that ECs, SMCs and fibroblasts could be isolated simultaneously and expanded in culture from a single and small vein sample (Grenier *et al*, 2003). However, majority of the cells in adult blood vessel are terminally differentiated, have limited proliferation potential and lose their function during *in vitro* expansion.

Nevertheless, alternative sources to differentiated vascular cells have been looked into to avoid the need of surgical harvest of autologous vessel segments and to improve upon the proliferation potential of ECs and SMCs. The use of stem cell is an exciting field of research for vascular regenerative medicine as these cells are capable of self-renewal and differentiation into functional.

MSCs are a promising cell type for regenerative medicine owing to their easy isolation and expansion, their multipotency and their low immunogenicity. MSC differentiation towards vascular phenotypes can be distinguished by the expression of experimentally identified specific markers or by functional assays. One unique advantage of MSCs is their potential for allogenic cell delivery in immunocompetent patients (Huang *et al*, 2008).

Adipose tissue is a stem cell source for ECs and SMCs. These cells can be harvested, multiplied and handled easily, efficiently and non-invasively. They have a proliferative capacity comparable to BM-MSCs, and morbidity to donors is considerably less, requiring only local anesthesia and a short wound healing time. Furthermore, human adipose stem cells have been shown to differentiate towards

endothelial lineage in the presence of VEGF (Cao *et al*, 2005).

Pericytes have recently been shown to express mesenchymal stem cell features. Their relative availability and multipotentiality make them a promising candidate for vascular regeneration. Pericytes were incorporated into bi-layered elastomeric poly(ester-urethane) urea scaffolds and the efficacy of the pericyte-seeded scaffold was studied *in vivo*. After implantation, pericyte-seeded grafs showed a significant higher patency rate than the unseeded control (He *et al*, 2010).

Progenitor cells such as Endothelial Progenitor cells (EPCs) show great potential for use in vascular regeneration. They are easy to obtain from the patient and are easy to expand in culture. EPCs are mainly located in bone marrow and could be mobilized into peripheral blood by certain growth factors, such as granulocyte macrophage colony stimulating factor (GM-CSF) or VEGF. EPCs could be also isolated from umbilical cord blood. EPCs can be expanded for over 20 passages without losing their differentiation potential. No significant differences have been found between EPCs derived from bone marrow, peripheral blood or cord blood in terms of cell proliferation and differentiation. Kaushal *et al* (2001) reported the isolation of EPCs from peripheral blood of sheep, their expansion and seeding on decellularized porcine iliac vessels to construct an engineered vascular graft. EPC-seeded grafts remained patent for 130 days as a carotid interposition graft in sheep. The EPC-explanted grafts exhibited contractile activity and nitric oxide mediated vascular relaxation that was similar to native carotid arteries.

AIMS

AMs seem to be a very promising scaffold in TE and can be considered as temporary inductive site-appropriate templates to support the growth, differentiation, and function of the parenchymal cell population of each organ. Nowadays, TE techniques are used both to develop tissue substitutes *ex vivo* and as reliable tool to investigate cell behaviour, differentiation and proliferation in 3-dimentional environments.

In this work, the following two different projects have investigated both potentialities using tissue-specific AMs:

1- influence of AMs on differentiation of kidney progenitor cells from amniotic fluid into mature renal cells;
2- AMs as biomaterial to develop vessel substitutes.

1- Evaluation of AMs as scaffold to promote kidney progenitor cells differentiation into mature renal cells

In order to better understand whether renal progenitors derived from human amniotic fluid could be suitable for future application in therapy, their behaviour and response when cultured onto AM system obtained from murine kidney (KAMs) have been investigated. To achieve this goal, renal progenitors were seeded onto KAMs and their proliferation and differentiation were evaluated by means of immunohystological analysis. Besides, to investigate renal progenitors behaviour in *in vivo* system, repopulated KAMs were intrarenal implanted into nude mice.

2- Evaluation of blood vessel substitutes composed by AMs and ECs

Although autologous vascular grafts and artificial materials have been used for reconstruction of small diameter (<5 mm) blood vessels, the poor availability of vessels and the occurrence of intimal hyperplasia and progressive atherosclerotic degeneration represent shortcoming of these vascular prostheses. Therefore, this study aimed to develop AM-based vascular grafts.

Rats received either only aorta AM or previously *in vitro* reendothelized AM as abdominal aorta interposition grafts (about 1 cm). After 1 and 3 months from

surgery, grafts were explanted and morphologically examined by scanning electron microscopy and Movat staining.

PART 1: Evaluation of AMs as scaffold to promote kidney progenitor cells differentiation into mature renal cells

MATERIALS AND METHODS

1. KIDNEY ACELLULAR MATRICES

a- Preparation

All the procedures described and animal protocols were approved by the IACUC at Children's Hospital Los Angeles. IACUC is the Institutional Animal Care and Use Committee on charge of overseeing CHLA animal programs, animal facilities and policies, ensuring appropriate care, ethical use and humane treatment of animals.

Both male and female C57BL/6 mice (3 months old) were sacrificed using CO_2 inhalation as recommended by IACUC and both kidneys were collected and rinsed with PBS.

Mouse kidney acellular matrices (KAMs) were prepared by the Meezan method (Meezan *et al*, 1975) with minor modifications. Whole kidneys were processed with distilled water for 72 h at 4˚C, 4% sodium deoxycholate (Sigma, St. Louis, MO, USA) for 4h and 2,000 kU deoxyribonuclease I (DNase-I) (Sigma) in 1 M NaCl (Sigma) for 3h. This treatment was repeated four times till the decellularization was completed.

b- Characterization of KAMs by histological and immunohistochemistry staining

The lack of cells or cell debris was confirmed histologically. Briefly, KAMs were fixed in 10% formalin, neutral buffer phosphate (Polysciences Inc.) for 2 h at RT and stored in 70% ethanol at 4°C until processing. Specimens were routinely processed as following described:

1h in 95% ethanol, twice;

1h in 100% ethanol, twice;

40 min in toluene;

overnight toluene/paraffin (50:50) and

2h paraffin

After this treatment, samples were included in paraffin in embedding cassette (Tissue-Tek) and prepared for sectioning. KAMs were cut in 5 μm sections with a Leica RM2235 microtome and let to dry on a slide warmer (Lab-Line) at 37 °C. Once dried, sections were ready to use for histological and immunostaining protocols. Lack of cells was confirmed by hematoxylin/eosin (H/E) and DAPI staining (VECTOR). Immunohistochemistry was performed as previously described using

anti-α2-laminin antibody (Santa Cruz) and anti-Collagen4 chain α1-2-3-4-5 antibody (Japan) in order to confirm the maintenance of ECM composition. The absence of cellular membrane residuals was confirmed using an anti-MHC I antibody (Abcam).

c- DNA content quantification

DNA content was quantified by DNeasy Blood & Tissue Kit (Qiagen), to check the acellularity of matrices. Briefly, KAMs were dried, minced, weight and digested overnight in Proteinase K. After lysis of the samples, DNA was separated from RNA through centrifugation using silica-gel columns. The obtained DNA was quantified by NanoDrop (Roche) measurements and it was separated in a 1.5% agarose/ethidium bromide gel and visualized using Blue/Orange Loading Dye (Promega, San Luis Obispo, CA).

2. CELL CULTURE

a- Expansion of human Amniotic Fluid (AF) Total Cell Population

Under Institutional Review Board approval of Children's Hospital Los Angeles, human amniotic samples were obtained from discarded amniocentesis fluid between 15 and 20 weeks of gestation. Samples with normal male karyotype and normal fetal ultrasound were collected from discarded cultures (Genzyme Pasadena, CA). Cells were expanded in Tissue Culture Dishes (BD Falcon, Franklin Lakes, NJ) with Chang's media (α MEM, 20% Chang B and 2% Chang C) (Irvine Scientific, Santa Ana, CA), L-Glutamine 20% of ES-FBS (Gibco/Invitrogen, Carlsbad, CA) and 1% of antibiotic (Pen/Strep, Gibco/Invitrogen, Carlsbad, CA).

b- Immunoseparation of CD24+OB-Cadherin+ population from whole Amniotic Fluid

A positive population from AF for both CD24 (Abcam) and OB-Cadherin (Abcam) was selected by two further immunoseparation using standard magnetic cell sorting (Miltenyi Biotech, Germany) following manufacturer's instructions. The total cell population was incubated with these two antibodies for 20 min at 4° C on shaking condition, followed by a second incubation with immunomagnetic microbeads for 15 min at 4° C followed by immunoseparation by MS columns (Miltenyi Biotech, Germany). Positive population was replated on Tissue Culture dishes with Chang's Media for subsequent expansion.

c- Analysis and characterization by immunohistochemistry of CD24+OB-Cadherin+ population

Between passages 4 and 5, cells previously seeded in chamber slides were fixed in 4% paraformaldehyde and stained as following described. Cells were treated for 30 min with hydrogen peroxidase, blocked in a solution of 3% BSA in PBS for 10 min at room temperature (RT) and incubated for 60 min with primary antibody. Bound primary antibodies were detected by a RT 30 min incubation with the suitable secondary antibody and revealed by DAB.

First antibody	Company	Diluition	Secondary antibody
Aq1	Santa Cruz	1:100	ImmPRESS Universal Antibody anti-rabbit Ig/anti-mouse Ig (Vector, MP 7500)
Aq2	Santa Cruz	1:50	Biotinylated Rabbit Anti-Goat IgG Antibody (Vector BA5000)
Peanut agglutinin	Vector	15 µg/mL	Biotinylated Rabbit Anti-Goat IgG Antibody (Vector BA5000)
Nephrin	Santa Cruz	1:50	Biotinylated Rabbit Anti-Goat IgG Antibody (Vector BA5000)
WT1	Santa Cruz	1:50	ImmPRESS Universal Antibody anti-rabbit Ig/anti-mouse Ig (Vector, MP 7500)
Podocalyxin	Invitrogen	1:50	ImmPRESS Universal Antibody anti-rabbit Ig/anti-mouse Ig (Vector, MP 7500)
Vimentin	Santa Cruz	1:50	Biotinylated Rabbit Anti-Goat IgG Antibody (Vector BA5000)
PDGFRβ	Abcam	1:50	ImmPRESS Universal Antibody anti-rabbit Ig/anti-mouse Ig (Vector, MP 7500)
VEGF	Abcam	1:50	ImmPRESS Universal Antibody anti-rabbit Ig/anti-mouse Ig (Vector, MP 7500)
Von Willebrand Factor	Santa Cruz	1:50	Biotinylated Rabbit Anti-Goat IgG Antibody (Vector BA5000)
Human nuclei	Acris	1:100	ImmPRESS Universal Antibody anti-rabbit Ig/anti-mouse Ig (Vector, MP 7500)

3. CD24+ OB-CADHERIN+ CELLS: KAM CULTURES

300 µm slides of KAMs were obtained using Vibratome (Leica). Sections were washed in PBS added with 10% Pen/Strep (Gibco) and placed at the bottom of a 25 cm^2 flask. Slides were kept in PBS overnight and placed in ES-FBS (Gibco/Invitrogen, Carlsbad, CA) 3 h before seeding.

Before seeding, CD24+ OB-Cadherin+ cells (10^6 cells/cm^2) were trypsinized, centrifuged at 1500 rpm for 5 min and labelled with the cell tracker CM-Dil (Molecular Probes), following manufacturer's instructions. CM-Dil is a membrane stain well retained through fixation/permeabilization processes and allows tracking of cells after injection. Briefly, cells were resuspended in 500 µl PBS and incubated with a working solution of Cm-Dil solution of 1 mg/mL for 5 min at 37°C, followed by an incubation of 15 min at 4°C. Cells were finally rinsed three times with PBS. The medium was mantained in agitation using a magnetic stirrer starting from 48 h from seeding. After 7, 14 and 21 days of culture, seeded KAMs were embedded in paraffin as previously described. The presence of CD24+ OB-Cadherin+ cells was confirmed by H/E and DAPI staining (VECTOR). To detect renal markers, immunohistochemistry was performed as above described.

The rating of proliferation was determined by PCNA Staining Kit (Invitrogen) following manufacture's instruction. Briefly, deparaffinized and rehydrated slides were treated with blocking solution for 10 min and with biotinylated mouse anti-PCNA primary antibody for 1 h. Rinsed sections were incubated with streptavidin-peroxidase solution and at last stained with chromogen DAB solution and hematoxylin.

3. *IN VIVO* EXPERIMENTS

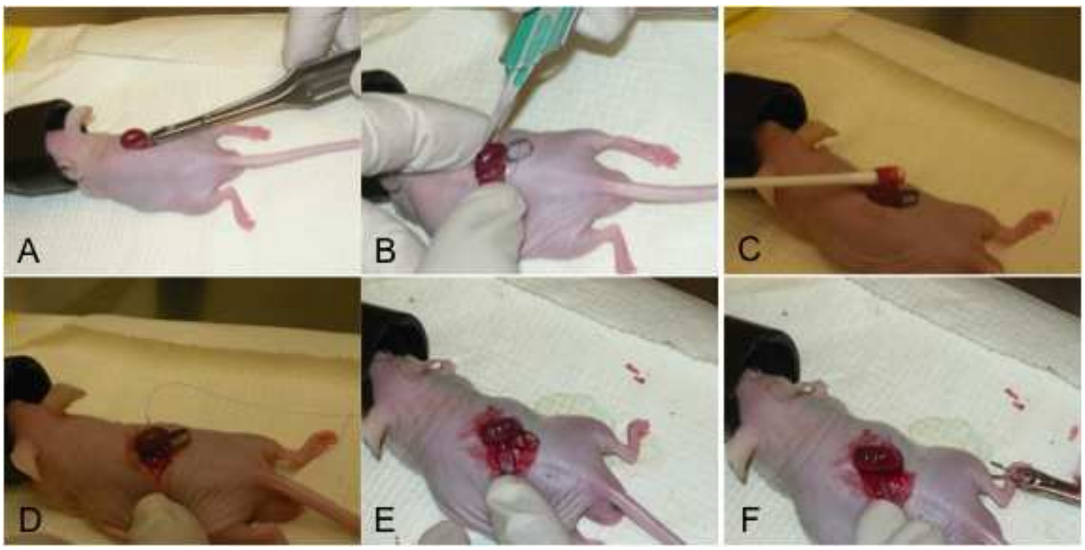
All the procedures described and animal protocols were approved by the IACUC at Children's Hospital Los Angeles.

Male 2 months old nude mice (J:Nu, Jackson Laboratories) were divided into 2 groups:

- group 1: received not seeded KAMs (n=4)
- group 2: received KAMs seeded with CD24+ OB-Cadherin+ cells 7 days after seeding (n=4)

The mice were carefully anesthetized using isofluorane inhalation method. Once satisfactory anesthesia was achieved, the mice were prepped using clorhexedine. A small approximate 1 cm dorsal lumbotomy incision was made, the kidney carefully delivered via the incision. A microvascular clamp was placed across the hilum preventing blood flow to the kidney. A small longitudinal incision was made along the lateral border of the kidney into the renal cortex. A 300 µm section of KAM repopulated or not with renal progenitor cells was inserted into this cortical defect.

The defect was closed with 6-0 interrupted polydioxanone suture. The microvascular clamp was removed. Once hemostasis was confirmed, the kidney was replaced into the retroperitoneum, the incision closed with polypropylene suture (Taper C-1, size 5-0, 90 cm), and the mice recovered from anaesthesia. The animals were maintained on a heating pad throughout the period of anaesthesia. Ophthalmic ointment was placed in each eye to prevent corneal drying; 0.1 mg/kg of buprenorphine was administered subcutaneously.



Kidney was delivered through a dorsal lumbotomy incision (A). The hilum was clamped and a small incision was made into the renal cortex (B). A 300 μm KAM slide was insert into the cortical defect (C). The incision was closed (D,E) and the microclamp removed (F).

Mice were sacrified after 1, 2, 3, 6 months by CO_2 inhalation and KAM implanted kidney collected and rinsed in PBS. Samples were embedded in paraffin and stained with H/E and DAPI (VECTOR) staining as previously described.

Immunohistochemistry was performed as above mentioned with minor modifications. Deparaffinized and rehydrated slides were treated for 30 min with hydrogen peroxidase, they were blocked in a solution of 3% BSA in PBS for 10 min at room temperature (RT) and incubated for 60 min with first primary antibody. Bound primary antibodies were detected by a RT 30 min incubation with the suitable secondary antibody and revealed by Red DAB. After washing, slides were incubated with the second primary antibody (against Human Nuclei or Human Mithocondria) for 1 h at RT and then with the suitable secondary antibody (Universal Antibody anti-rabbit Ig/anti-mouse Ig,Vector, MP 7500). At last, stain was developed with DAB and nuclei stained with hematoxylin.

Collagen fibers were detected by Masson staining Kit (Sigma), which was performed following manufacture's instruction. Briefly, deparaffinized and rehydrated slides were treated with Bouin's solution for 1 h at 56°C and washed in running tap water. Sections were stained in working Weigert's Iron Hematoxylin Solution for 5 min. Rinsed slides were treated with Biebrich Scarlet-Acid Fuchsin and then placed in Phosphomolybdic/phosphotungstic acid solution for 5 min. Sections were treated with Aniline Blue Solution for 5 min and placed in acetic acid for 2 min. Then, slides were dehydrate to xylene and mounted with balsam.

RESULTS

1. KIDNEY ACELLULAR MATRICES

Four cycles of detergent-enzymatic treatment were needed to completely remove cells, as confirmed by H/E (Fig. 1 B) and DAPI staining (Fig. 1 D). KAMs maintained the structure of native kidney (Fig. 1A), in particular the glomerular basement membrane was well visible (Fig. 1B).

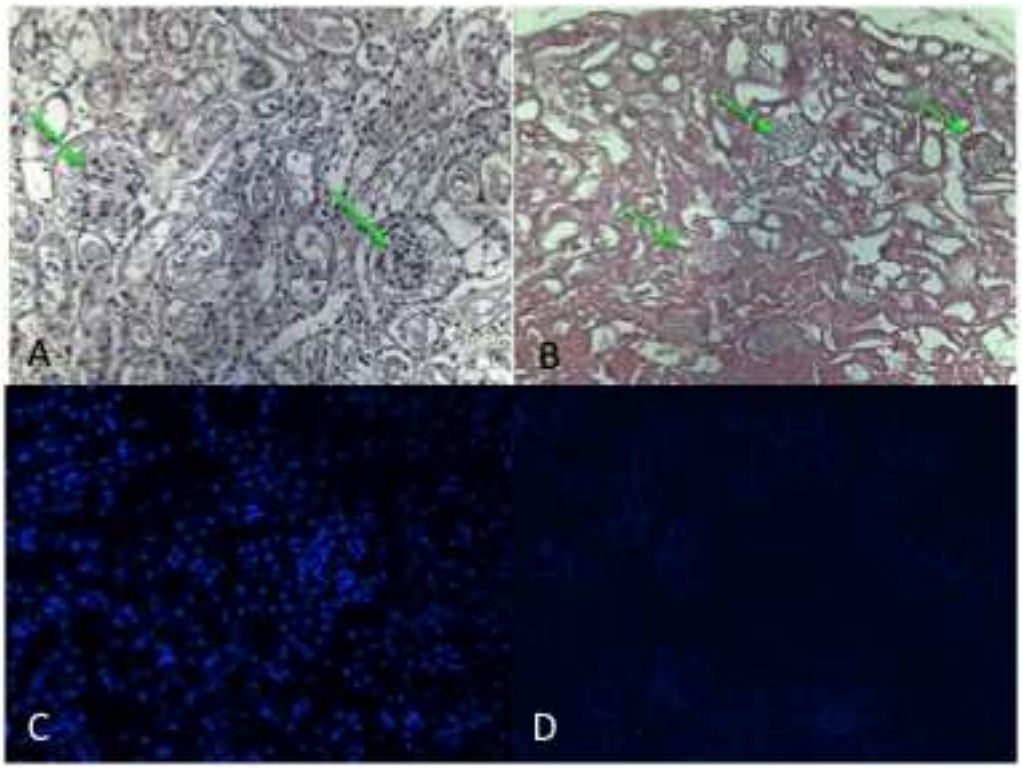


Figure 1. H/E (A, B; magnification x200) and DAPI (C, D; magnification x200) staining before (A, C) and after (B, D) 4 cycles of detergent-enzymatic treatment. Arrows indicate glomerular basement membranes.

Immunohystochemistry revealed the absence of cell membrane antigens MHC I in KAMs (Fig. 2 B), normally present in native tissue (Fig. 2 A).

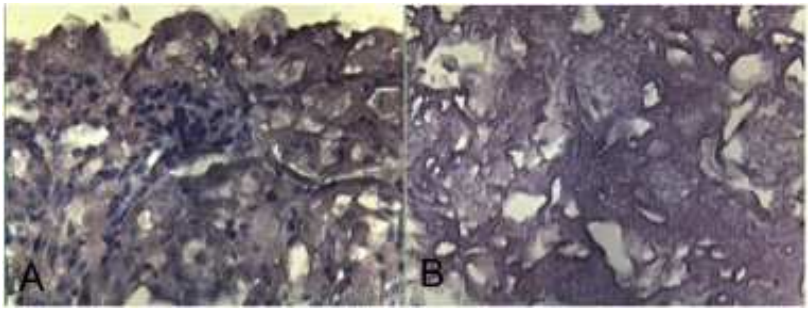


Figure 2. Immunohystochemistry performed using anti-MHC I antibody before (A) and after 4 cycles of detergent-enzymatic treatment (B). Magnification x400

The maintenance of ECM proteins was confirmed by immunohistoschemistry performed using anti-collagen type IV chains I (Fig. 3 A, D), II (Fig. 3 B, E), III (Fig. 3 C, F), IV (Fig. 3 G, L), V (Fig. 3 H, M) and laminin α2 (Fig. 3 I, N) antibodies. KAMs (Fig. 3 D, E, F, L, M, N) showed all glomerular basement membrane proteins, as native kidney (Fig. 3 A, B, C, G, H, I).

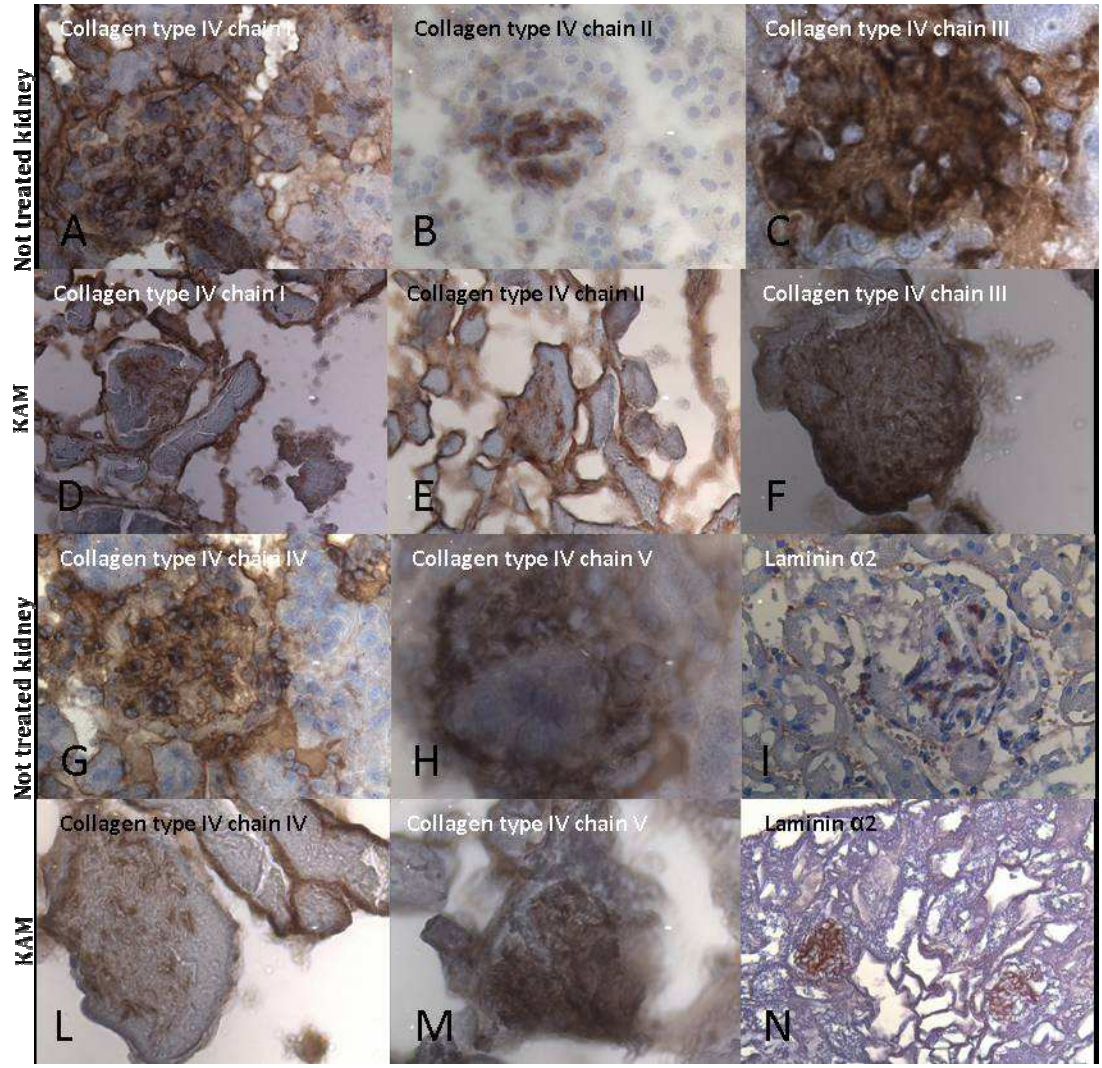


Figure 3. Immunoistochemistry of native kidney (A, B, C, G, H, I) and KAM (D, E, F, L, M, N) performed using anti-collagen type IV chain I (A, D), II (B, E), III (C, F), IV (G, L), V (H, M; magnification x1000) and laminin α2 antibodies (I, N; magnification x400).

The amount of residual DNA content after decellularization was quantified as shown in Fig. 4. Although bands of DNA were not visible after separation on 1.5% agarose gel (Fig. 4 A), 30.27 ± 11.3 ng DNA/mg dry weight were determined in KAMs compared to 2544.43 ± 97.67 ng DNA/mg dry weight found in native kidney (Fig. 4 B).

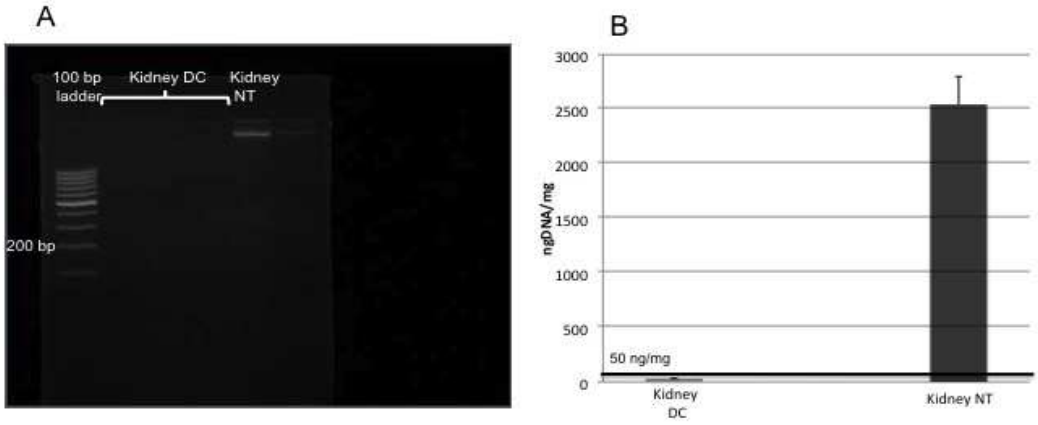


Figure 4. Residual DNA content detected by 1.5% agarose/ethidium bromide gel (A) and DNeasy Blood & Tissue Kit (B, n= 5).

2. CELL CULTURE

The cell population positive for CD24 and OB-Cadherin was successfully isolated from whole AF and cultured under the conditions described above. Selected cells presented a typical fibroblast shape (Fig. 5).

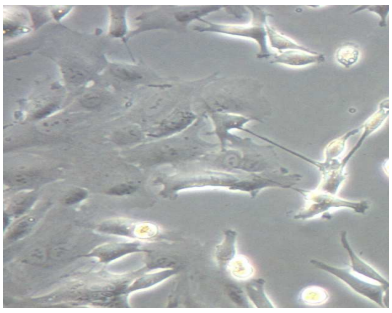


Figure 5. Phase contrast microscopy of CD24+ OB-Cadherin+ cell cultures (magnification x200).

CD24+ OB-Cadherin+ cells were previously characterized by RT-PCR for early and mature kidney markers (Da Sacco *et al*, 2010). Cell extracts contained mRNAs for Aquaporin 1 (Aq1), Occludin, Nephrin, Lim1, Pax-2 (paired box gene 2), GDNF (Glial Cell Line-derived Neurotrophic Factor), and Zo-1 (Zonula Occludens 1).

CD24+ OB-Cadherin+ cells expressed Podocalyxin (Fig. 6 A) and VEGF (Fig. 6 B), while WT-1 (Wilms tumor protein-1, Fig. 6 C), Aq1 (Fig. 6 D), Aq2 (Fig. 6 E) and Peanut Agglutinin (Fig. 6 F) were not detected.

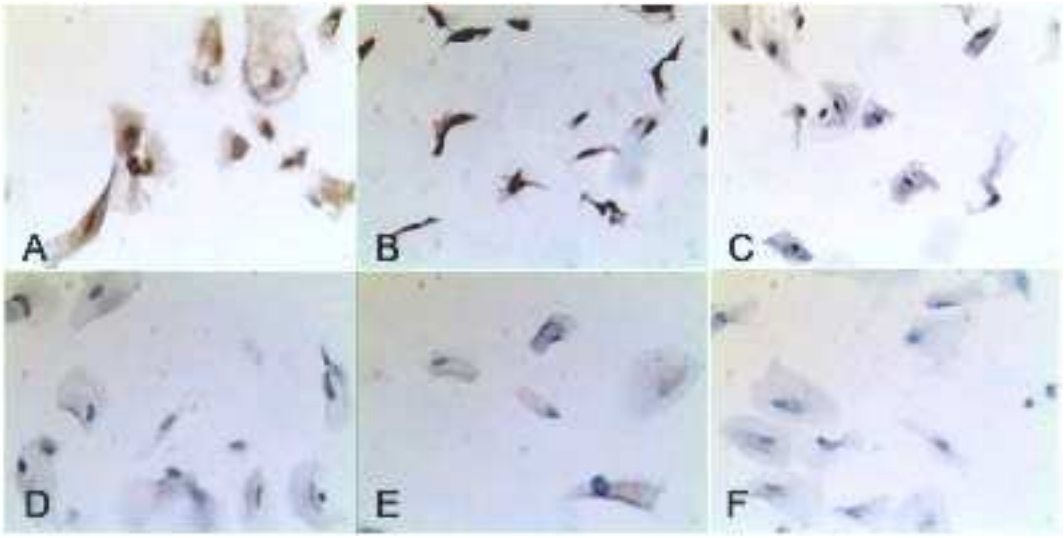


Figure 6. Immunohistochemistry of CD24+ OB-Cadherin+ cells Podocalyxin (A), VEGF (B), WT-1 (C), Aq1 (D), Aq2 (E) and Peanut Agglutinin (F) expression (magnification x200).

3. CD24+ OB-CADHERIN+ CELLS/KAM CULTURES

CD24+ OB-Cadherin+ cells were seeded onto 300 μm KAM slides. Cells engrafted the biomaterial and were able to migrate inside it, as shown by DAPI staining 7 (Fig. 7 A), 14 (Fig. 7 B) and 21 (Fig. 7 C) days after seeding.

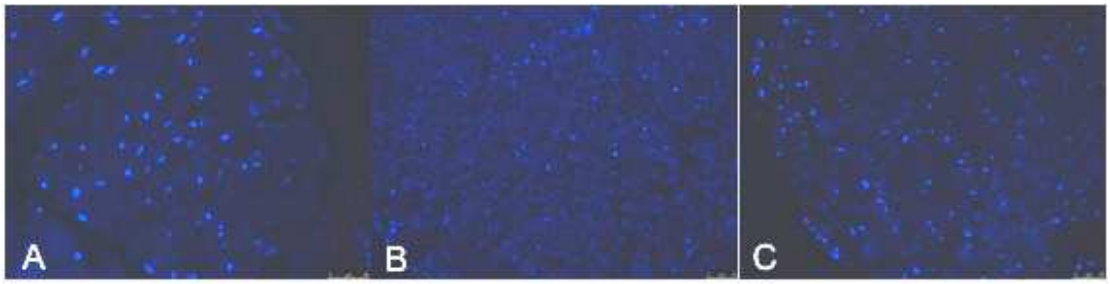


Figure 7. DAPI staining of CD24+ OB-Cadherin+ cell/KAM cultures 7 (A), 14 (B) and 21 (C) days after seeding (magnification x100)

Cell engraftment was shown by SEM analysis (Fig.8). In particular, renal progenitors were able to rearrange and merge (Fig. 8 B) and shown foot processe buds (Fig. 8 C).

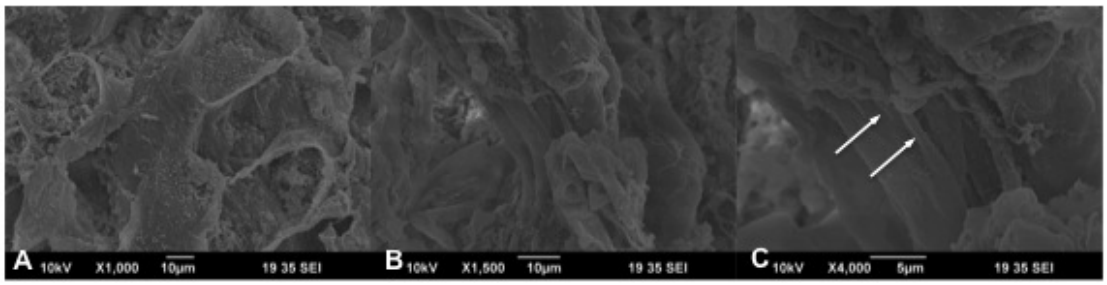


Figure 8. SEM analysis of CD24+ OB-Cadherin+ cell/KAM cultures 7 days after seeding. Arrows indicate foot processe buds.

Besides, PCNA staining demonstrated that CD24+ OB-Cadherin+ cells were able to proliferate till 14 d after seeding (Fig. 9 A, B), on the contrary, cell growth seemed to stop at 21 days, as determined by lack of staining (Fig. 9 C).

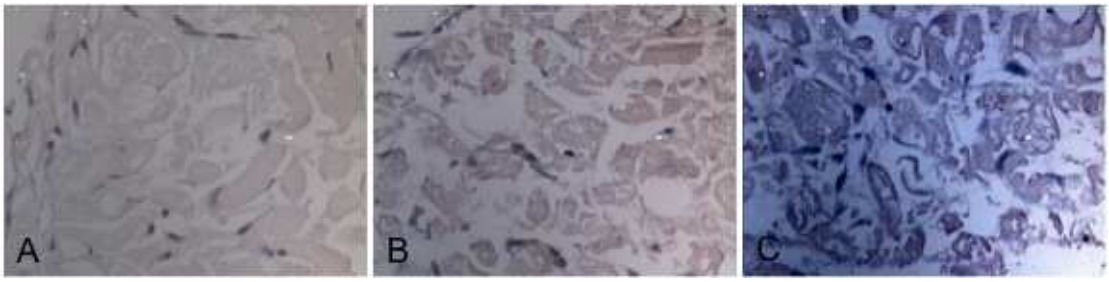


Figure 9. PCNA staining of CD24+ OB-Cadherin+ cells/KAM culture 7 (A), 14 (B) and 21 (C) days after seeding (magnification x400).

CD24+ OB-Cadherin+ cells seeded onto KAMs were characterized by immunohistochemistry. At each endpoint renal progenitors expressed VEGF, Podocalyxin and Aq1 (Fig. 10), but not Aq2, Peanut Agglutinin and WT1 (data not shown).

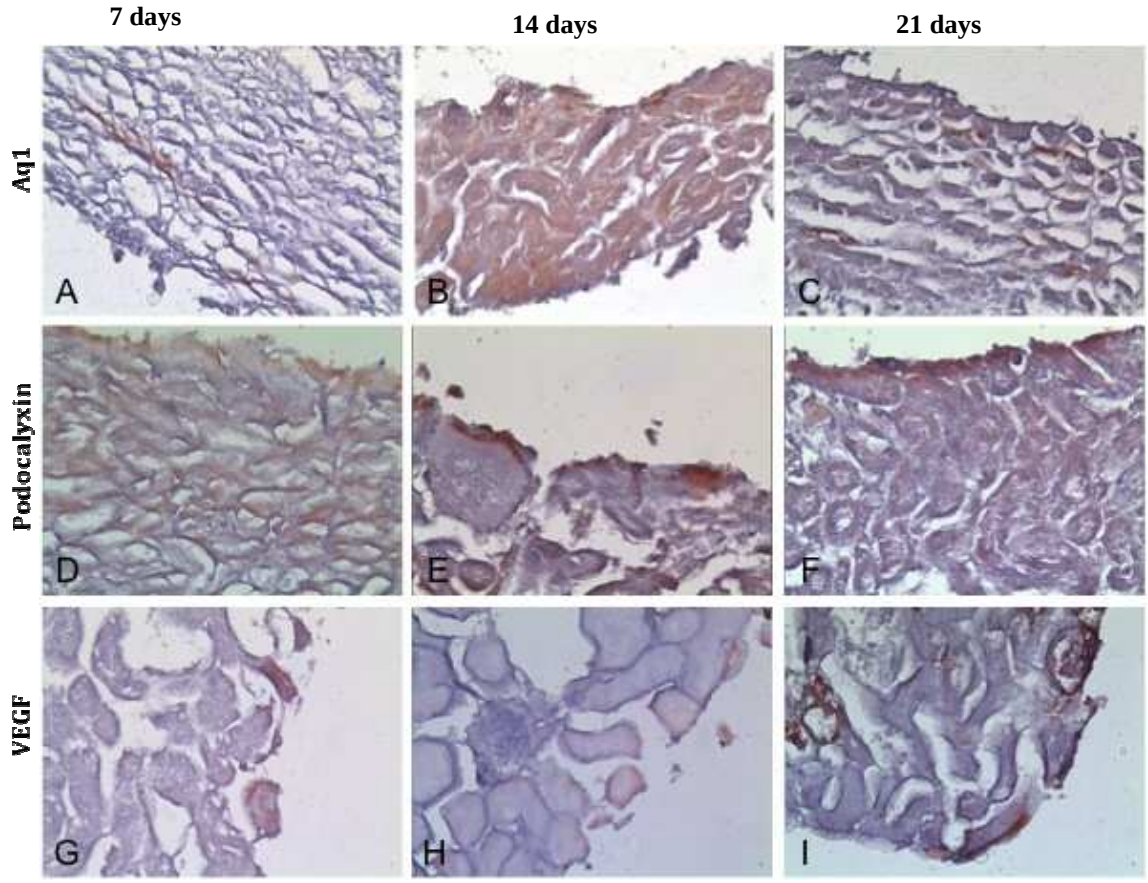


Figure 10. Immunohistochemistry of CD24+ OB-Cadherin+ cell/KAM cultures performed using anti-Aq1 (A, B, C), -Podocalyxin (D, E, F), -VEGF (G, H, I) antibodies 7 (A, D, G), 14 (B, E, H) and 21(C, F, I) days after seeding (magnification x400).

4. *IN VIVO* EXPERIMENTS

KAM alone and KAM plus cells were intrarenal implanted in nude mice. Group 1 received only KAM while group 2 received KAM seeded with CD24+ OB-Cadherin+ cells, 7 days after seeding. At each endpoint, in both groups KAMs were largely repopulated by host cells, presented tubular-like structures and blood vessels, as shown by H/E staining (Fig. 11).

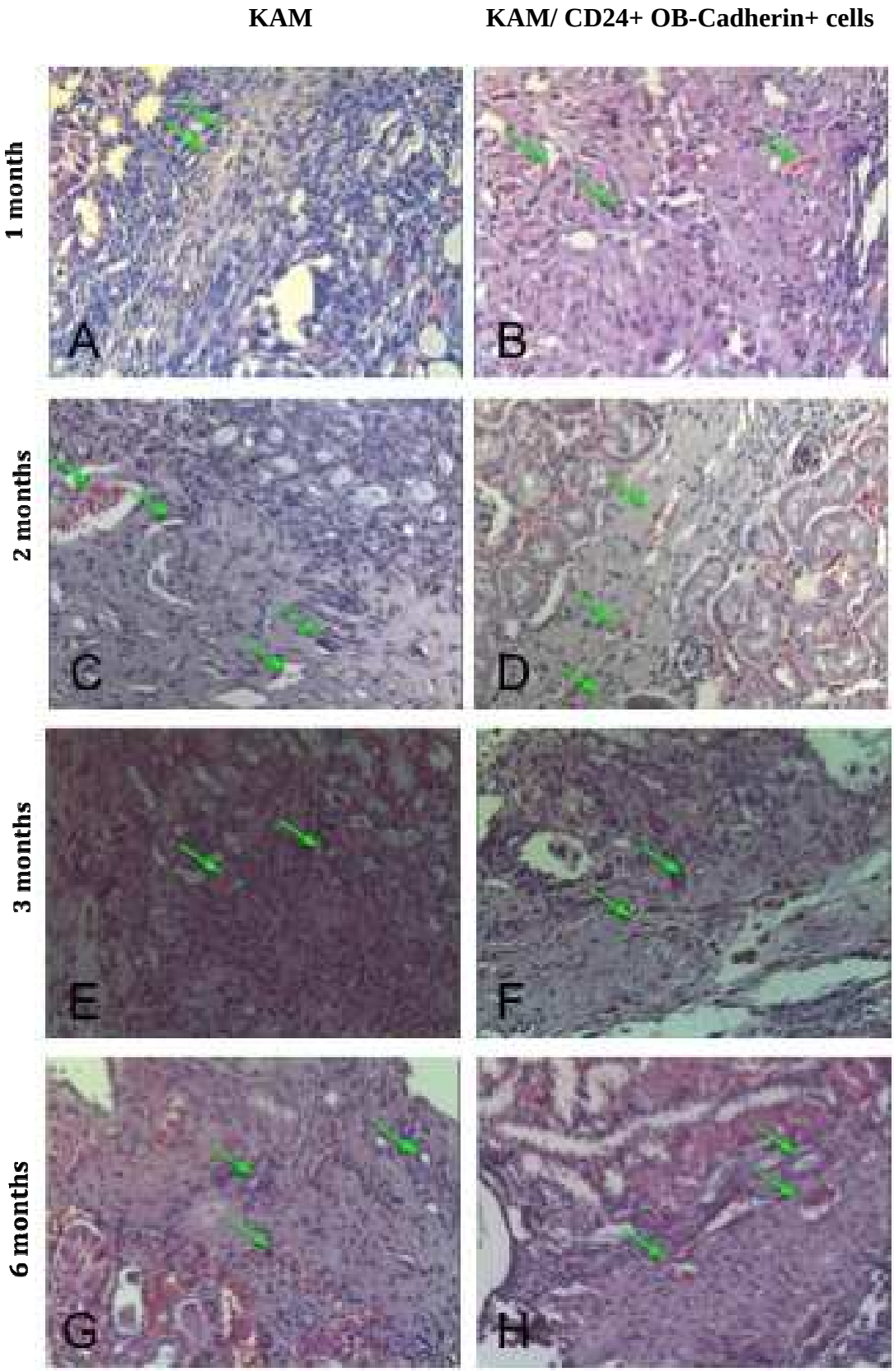


Figure 11. H/E staining. A, C, E, G: group 1; B, D, F, H: group 2. A, B: 1 month; C, D: 2 months; E, F: 3 months; G, H: 6 months (magnification x200). Arrows indicate tubular-like structures and blood vessels.

One month after surgery both groups presented endothelial cells and blood vessels inside the graft, as demonstrated by immunohistochemistry performed using anti-von Willebrand factor (Fig. 12 A, B) and CD31 (Fig. 12 C, D) antibodies.

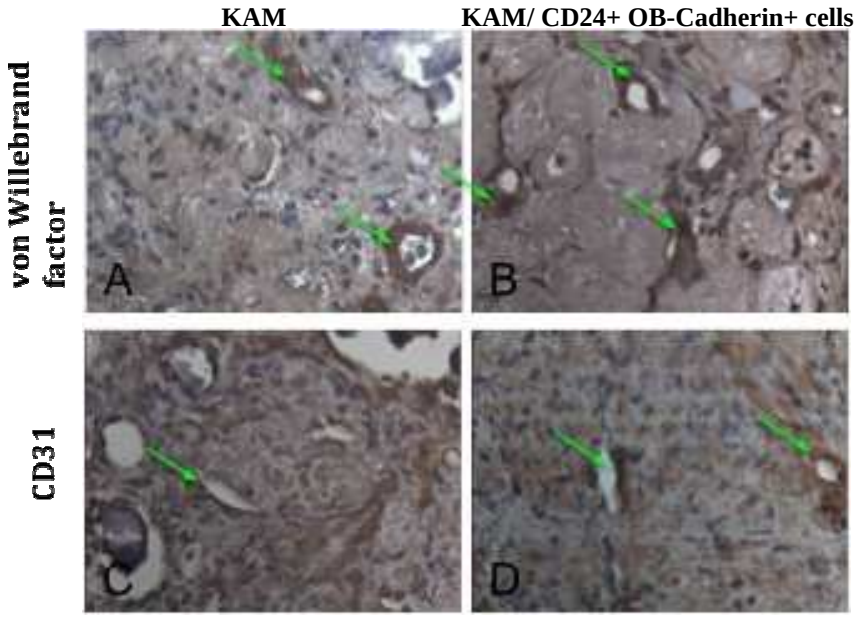


Figure 12. KAM (A, C) and KAM plus cells (B, D) immunohistochemistry performed using anti-von Willebrand factor (A, B) and anti-CD31 (C, D) antibodies (magnification x400). Arrows indicate positive staining.

In group 2, the permanence of CD24+ OB-Cadherin+ cells was revealed by human nuclei staining. Human cells were still present at all timepoints, but their number seemed to decrease over the time (Fig. 13).

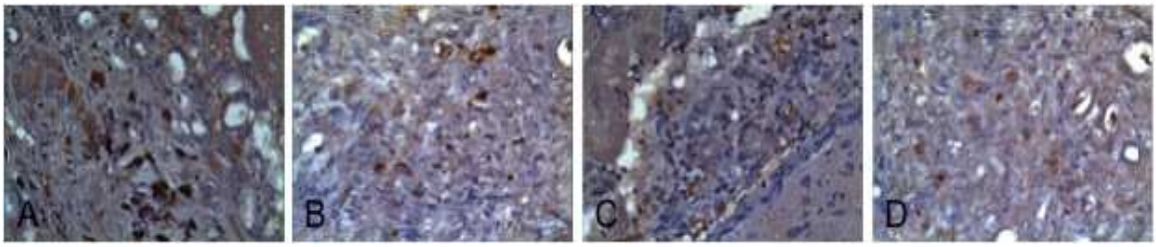


Figure 13. Immunohistochemistry performed on KAM plus cells, 1 (A), 2 (B), 3 (C), 6 (D) months after surgery using anti-human nuclei antibody (magnification x400).

Immunohistochemistry performed against different mature renal markers were performed in order to evaluate CD24+ OB-Cadherin+ cells differentiation.

Aq1, Aq2 and Peanut Agglutinin were considered as markers of differentiation into tubular cells.

In group 1, Aq1 positive cells were present only 6 months after intrarenal implantation (Fig. 14 G). On the contrary, in group 2, this marker was visible at each endpoint. A double staining performed using both anti-human nuclei and anti-Aq1

antibodies demonstrated that Aq1 was expressed by both CD24+ OB-Cadherin+ cells and host cells migrated inside the scaffold (Fig. 14 B, D, F, H).

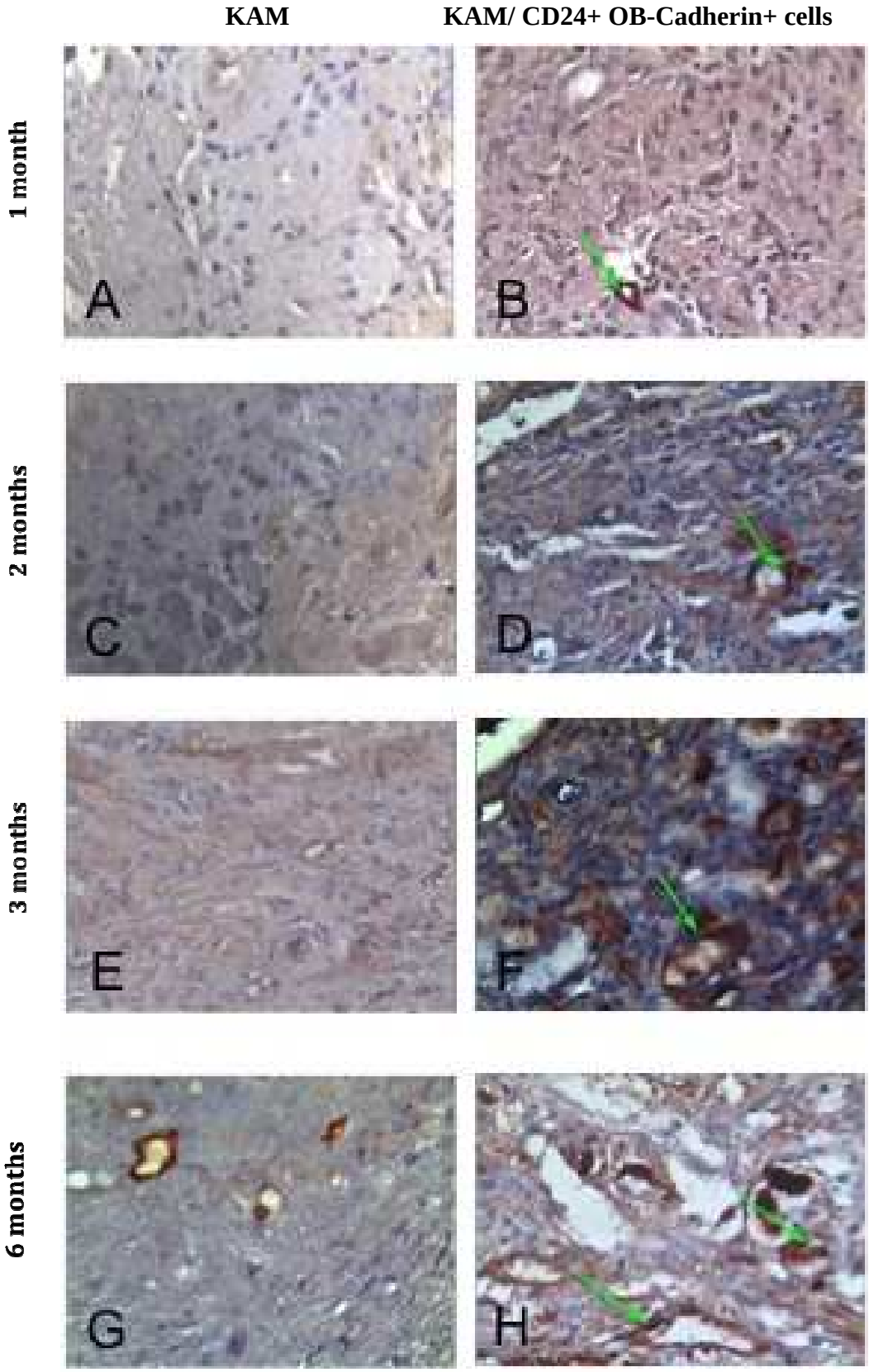


Figure 14. Immunohistochemistry performed using anti-Aq1 antibody on KAM graft (A, C, E, G) and KAM plus CD24+OB-Cadherin+ cells (B, D, F, H) 1 (A,B), 2 (C, D), 3 (F, G), 6 (H, I) months after surgery. B, D, F and H were obtained using both anti-Aq1 and ant-human nuclei antibodies (magnification x400). Arrows indicates double staining positive cells.

Similar results were obtained for Aq2. Indeed, the protein was immunodetected in both human and host cell in KAM plus cells starting from 1 month, while staining was negative in KAM (Fig. 15).

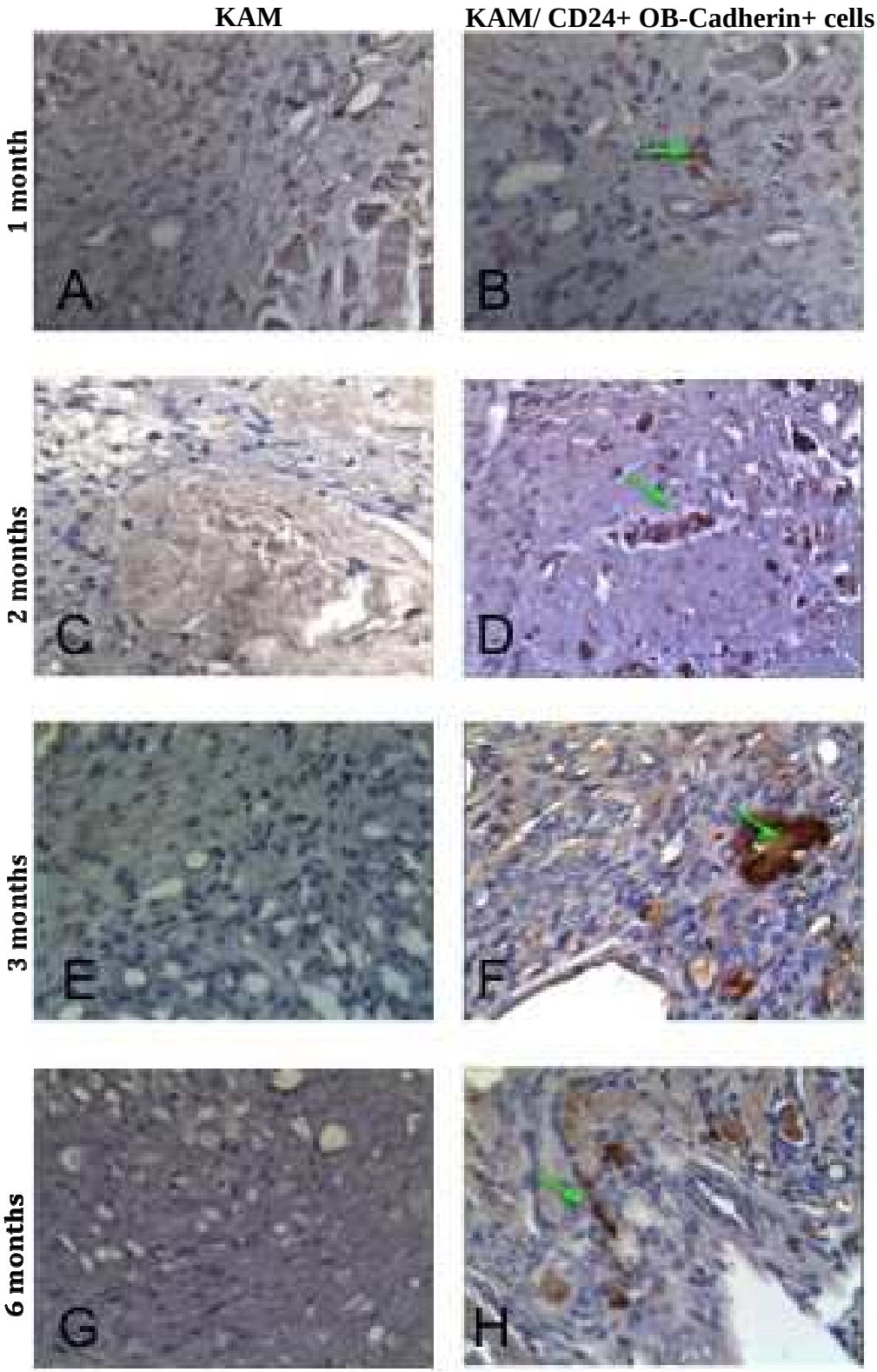


Figure 15. Immunohistochemistry performed using anti-Aq2 antibody on KAM (A, C, E, G) and KAM plus CD24+OB-Cadherin+ cells (B, D, F, H) 1 (A,B), 2 (C, D), 3 (F, G), 6 (H, I) months after surgery. B, D, F and H were obtained using both anti-Aq2 and anti-human nuclei antibodies (magnification x400). Arrows indicate double staining positive cells.

Besides, human Aq2 positive cells were shown to migrate into the native kidney and integrate into native tubuli (Fig. 16).

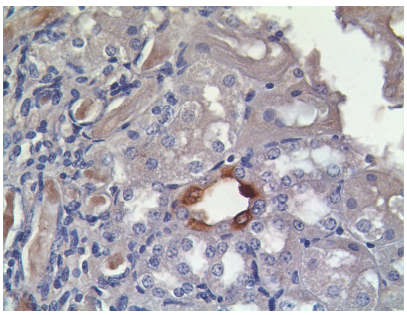


Figure 16. Aq2-human nuclei double staining of KAM plus CD24+OB-Cadherin+ cells 6 months after surgery (magnification 400x).

In KAM grafts Peanut Agglutinin was not detected (Fig. 17 A, C, E, G), whereas only murine cells expressed this marker in KAM plus CD24+OB-Cadherin+ graft (Fig. 17 B, D, F, H).

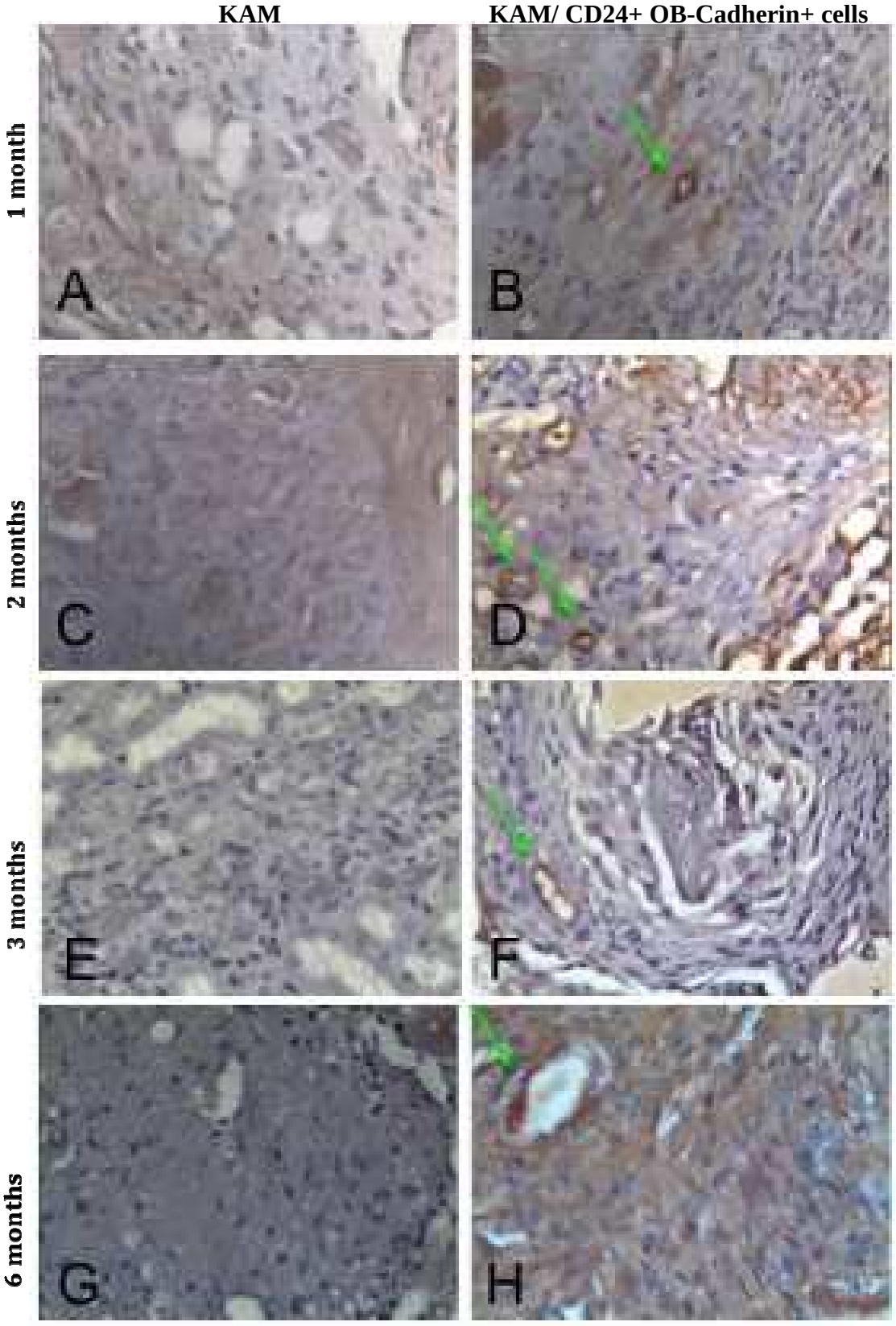


Figure 17. Immunohistochemistry performed using anti-Peanut Agglutinin antibody on KAM (A, C, E, G) and KAM plus CD24+OB-Cadherin+ cells (B, D, F, H) 1 (A,B), 2 (C, D), 3 (F, G), 6 (H, I) months after surgery. B, D, F and H were obtained using both anti-Peanut Agglutinin and anti-human nuclei antibodies (magnification x400). Arrows indicate Peanut Agglutnin positive staining.

To verify podocyte differentiation, immunoreactivity against Podocalyxin and WT1 antibodies was evaluated.

In group 2, both human and murine cells expressed Podocalyxin (Fig 18 B, D, F, H) and WT1 (Fig 19 B, D, F, H) 1 month after implantation, while in group 1 Podocalyxin appeared at 3 months (Fig. 18 E, G) and WT1 at 6 months (Fig. 19 E, G).

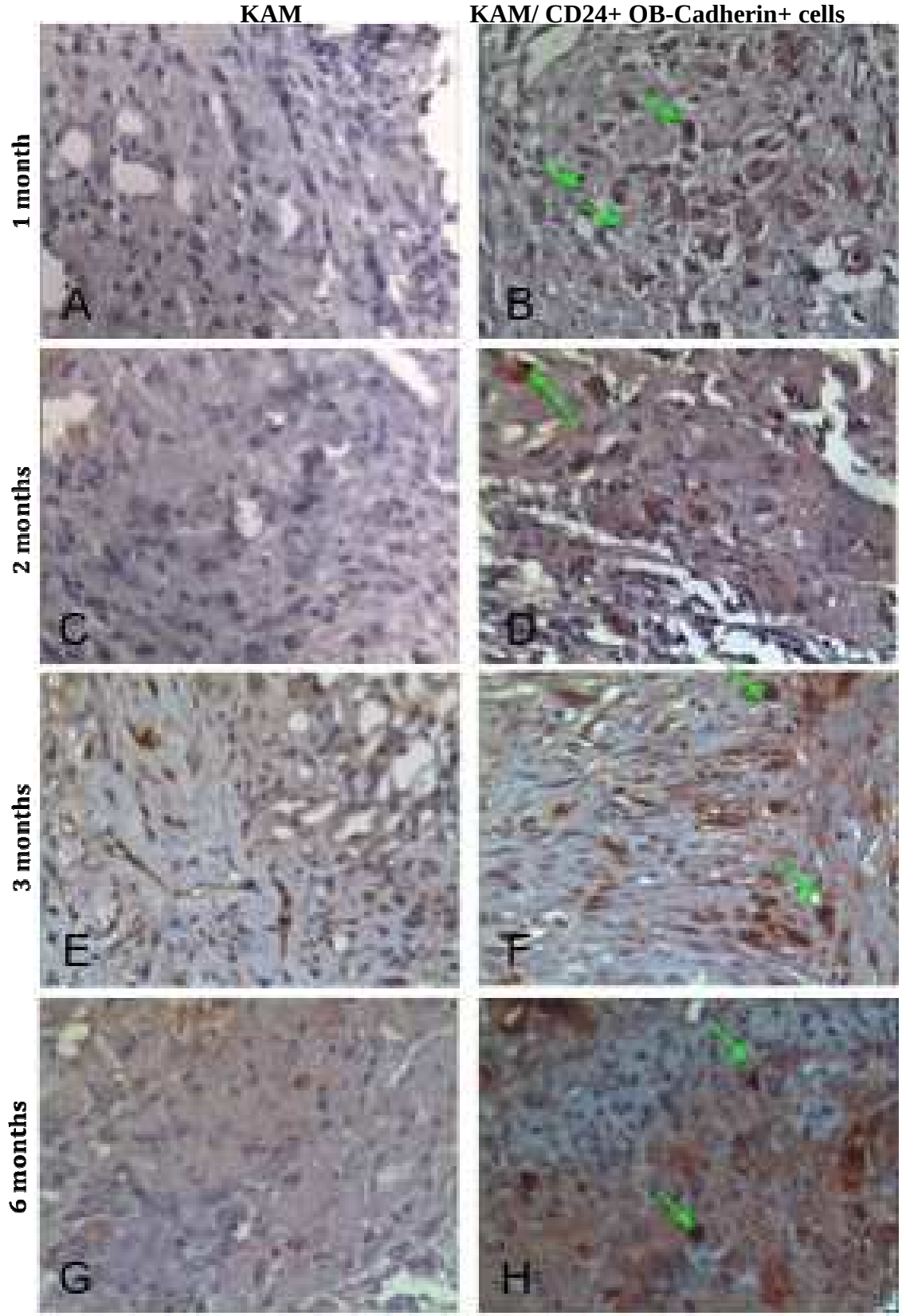


Figure 18. Immunohistochemistry performed using anti-Podocalyxin antibody on KAM (A, C, E, G) and KAM plus CD24+OB-Cadherin+ cells (B, D, F, H) 1 (A,B), 2 (C, D), 3 (F, G) and 6 (H, I) months after surgery. B, D, F and H were obtained using both anti-Podocalyxin and anti-human nuclei antibodies (magnification x400). Arrows indicated double staining.

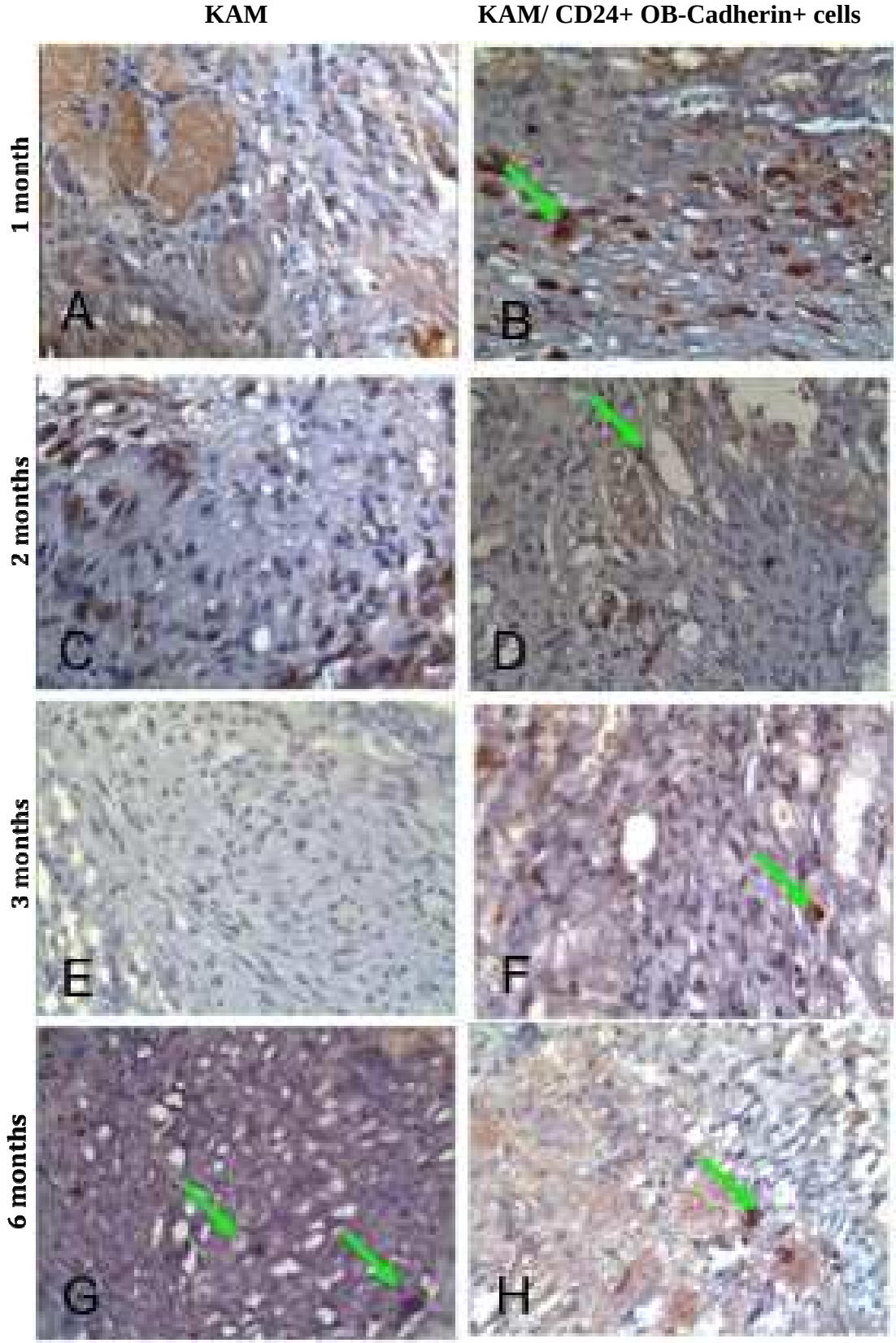


Figure 19. Immunohistochemistry performed using anti-WT1 antibody on KAM (A, C, E, G) and KAM plus CD24+OB-Cadherin+ cells (B, D, F, H) 1 (A,B), 2 (C, D), 3 (F, G) and 6 (H, I) months after surgery. B, D, F and H were obtained using both anti-WT1 and anti-human mitochondria antibodies (magnification x400). Arrows indicate WT1 staining in KAM and WT1-Human Mitochondria double staining in KAM plus cells graft.

DISCUSSION

This work has demonstrated that KAMs are able to support proliferation and differentiation of amniotic fluid renal progenitor cells.

To obtain KAMs various decellularization protocols have been tested. Detergents, such as SDS and Triton X100 (Nakayama *et al*, 2010; Orlando *et al*, 2012), appeared not suitable because they disrupted collagen structure and lead to incomplete cell removal (data not shown). On the contrary, using Meezan method with minor modifications, KAMs maintained the native architecture and composition. In particular, both glomerular basement membrane and the filtering membrane protein composition were well preserved. The decellularization treatment satisfied established parameters for DNA content: lacking of visible nuclei after DAPI staining, having less than 50 ng/mg AM dry weight and having all residual DNA fragments be less than 200 base pair in size (Crapo *et al*, 2011; Reing *et al*, 2010). The lack of antigenic epitopes associated with cell membranes demonstrated the non-immunogenicity, which is necessary to avoid, or at least minimize, adverse immune responses by allogeneic or xenogeneic recipients.

KAMs revealed to be a suitable scaffold for renal progenitor cells. Indeed, cells engrafted the biomaterial and were able to migrate inside it.

Cells seeded onto KAMs proliferated till 14 days from seeding and maintained the expression of VEGF and Podocalyxin, markers used for podocyte identification. Furthermore, SEM analysis shown that cells seeded into KAM presented foot processes buds, which are peculiar podocyte features. These results agree to Leapley *et al* (2009), that demonstrated that KAM allows fetal cortical renal cells to proliferate maintaining podocyte progenitor phenotype.

Without the use of pro-differentiation agents, KAMs have been shown to drive ESC differentiation towards the early steps of renal differentiation (Ross *et al*, 2009). On the contrary, in this study, when seeded into KAM without any inductive factor, renal progenitors from amniotic fluid expressed mature proximal tubular marker, such as Aq1. More investigations will be needed to demonstrate whether Aq1 positive cells could express other proximal tubular markers, as CD13 and Angiotensinogen.

To further evaluate the differentiative potential of KAMs, grafts composed of KAM with or without cells were intrarenal implanted into nude mice. Host cells largely repopulated KAMs grafts. Murine cells migration confirmed that KAMs presented biocompatible

structural features. However, migrated cells did not express tubular or podocyte markers till 3-6 months after surgery, although at earlier timepoints most of them presented PDGFRβ (data not shown), demonstrating their mesangial origin. The delayed migration of cells expressing tubular or podocyte markers could be promoted by KAM degradation products that have been shown to have chemotactic and mitogenic effects on cells (Badylak *et al*, 2011).

Grafts composed of KAMs plus cells shown the presence of differentiation markers just 1 month after surgery. Differentiated cells were both murine and human. It can be supposed that murine cells migrated earlier than in KAM grafts because renal progenitors remodel and degrade KAM that may release soluble molecules regulating cells motility. Besides, renal progenitors could produce a variety of paracrine factors that can enhance cell migration and engraftment (Li *et al*, 2010). Intrarenal environment, as expected, promoted renal progenitor cell differentiation. CD24+ OB-Cadherin+ cells were shown to express proximal tubular (Aq1), collector duct (Aq2) and distal tubular (Peanut Agglutinin) markers. Moreover, they expressed WT1, commonly present in developing renal vesicles and podocytes, as well as VEGF and Podocalyxin. The co-expression of the three markers would confirm the differentiation of cells into podocyte lineage. CD24+ OB-Cadherin+ cells potentiality to differentiate into different renal mature cells was expected because CD24 and OB-Cadherin are co-expressed by uninduced Metanephric Mesenchyme, the embryonic layer that gives rise to the entire nephron in adult life.

Interestingly, human CD24+ OB-Cadherin+ cells were also able to migrate outside the scaffold and integrate into host kidney. In particular, as native papilla cells, Aq1 positive human cells were shown to integrate into host renal tubules (Curtis *et al*, 2008).

Overall, these results demonstrated that KAMs obtained by Meezan protocol, maintaining ECM structure, could be a valuable tool to study both *in vitro* and *in vivo* differentiation towards renal cell phenotype.

Furthermore, these preliminary data encourage us to evaluate whether this approach could be useful to enhance the function of hypoplastic kidneys.

PART 2: Evaluation of blood vessel substitutes composed by AMs and ECs

MATERIALS AND METHODS

1. AORTA ACELLULAR MATRICES

a- Preparation

All the procedures described and animal protocols were approved by the CEASA at University of Padova. CEASA is University Ethical Animal Use Committee on charge of overseeing Padova University animal programs, animal facilities and policies, ensuring appropriate care, ethical use and humane treatment of animals.

Male Lewis rats (3 months old) were sacrificed using CO_2 inhalation as recommended by IACUC and abdominal aorta segments were collected and rinsed with PBS.

Aorta acellular matrices (AAMs) were prepared by the Meezan method (Meezan *et al*, 1975) with minor modifications. Briefly, aortas were processed with distilled water for 72 h at 4˚C, 4% sodium deoxycholate (Sigma, St. Louis, MO, USA) for 4 h and 2,000 kU deoxyribonuclease I (DNase-I) (Sigma) in 1 M NaCl (Sigma) for 3 h. This treatment was repeated twice times till the decellularization was completed. Between the two decellularization cycles, aortas were treated with Collagenase IV (0.05% in PBS) at 37°C for 1 min in order to remove the endothelium.

b- Evaluation of AAMs by histological and immunohistochemistry staining and Scanning Electron Microscopy

Histological samples and analysis were performed as previously described. Lack of cells was confirmed by hematoxylin/eosin (H/E) and DAPI staining (VECTOR).

Movat pentachromic staining was performed following manufacturer's instruction (Diapath). Briefly, deparaffinized sections were treated with alcian blue, which stains mucin and background. Elastic fibers were purple-black stained by hematoxilyn, collagen was yellow by treatment with fuchsin and alcoholic safran solution stained black nuclei.

Immunohistochemistry was performed as previously described using anti-MHC I and II antibody (Abcam) to confirm the absence of cellular membrane residuals.

AAM samples were fixed with 4% gluteraldehyde in 0.1 M cacodylate buffer (pH 7.2) and dehydrated. After critical point drying and gold sputtering, they were examined by a scanning electron microscope (SEM; Stereoscan-205 S, Cambridge, UK).

2. CELL CULTURES

a- Skin microvascular ECs: isolation and characterization

Dermis biopsies were harvested from male Lewis rats (3-5 months old). Specimens were rinsed in PBS, minced and subsequentially digested enzimatically with 0.25% Collagenase B (Roche), and 0.25% Dispase II (Roche), for 1 h at 37°C. Not digested dermis was removed using a cell strainer (100 μm, BD Falcon). Cells were seeded in Tissue Culture Petri Dishes (BD Falcon) previously conditioned with Fibronectin (1 $\mu g/cm^2$, BD Biosciences) in MV2 endothelial media (PromoCell). When cells were almost confluent, they were detached and immunoseparated using Dynabeads M-450 (Invitrogen) previously conditioned with a monoclonal antibody against CD31 (AbD Serotec) following manufacter's instruction. Briefly, cells were incubated with magnetic beads previously conditioned with the antibody for 30 min at 4 °C (5 beads for each cell). A magnetic field was applied to the cells for 5 min. CD31-positive cells tied to the beads through the antibody remained attached to the magnet. CD31-negative cells were discarded. ECs were replated in Tissue Culture Petri Dishes previously conditioned with MV2 endothelial media. Only cells between passages 1 and 4 were used.

The maintenance of endothelial markers was confirmed by immunofluorescence analysis using antibody against CD31 (Santa Cruz Biotecnology, 1:100) and von Willebrand Factor (1:400, Abcam). Briefly, cells were fixed with 4% formalin. Then, they were incubated for 1 h with 10% horse serum in PBS followed by 1 h treatment with the solution of the primary antibody. After rinsing with PBS, cells were treated with the secondary antibody for 30 min and with Flurescein Avidin DCS 1:500 in HEPES 10 mM and NaCl 0.15 M for 10 min. Nuclei were recognized by DAPI staining.

b- ECs/AAM cultures

ECs ($4x10^5/cm^2$) were seeded onto the luminal surface of AAMs, previously treated with FCS for 3 h. Cultures were maintained for 72 h in MV2 media and then fixed for histological analysis or *in vivo* implanted.

3. *IN VIVO* EXPERIMENTS

Female Lewis rats (3 months old) were carefully anesthetized using isofluorane inhalation method (3% isofluorane carried by oxygen, 1 L/min). Each recipient animal underwent a median laparotomy incision. After peritoneal incision, animals

received 5 mg/mL Tramadol (Contramal®) intraperitoneally. After systemic heparinisation (70 U/Kg) and proximal and distal clamping of the abdominal aorta, a segment of 1 cm length was removed. The same segment was replaced by only AAM (n=7) or AAM plus ECs (n=9). Anastomoses were performed with 8-0 or 9-0 interrupted polypropylene suture (Ethicon). Once the incision closed, rats were recovered from anaesthesia. Animals received an antibiotic (Terramicina ®, 60 mg/kg) on the 3rd and 6th day after surgery and analgesics (Contramal ®, 5 mg/kg), for 3 days postoperatively. No anticoagulants or antiplatelets were administered post-operatively.

Animals were sacrificed by Phenobarbital administration either 1 (n=8) or 3 months (n=8) after implantation. Arterial conduits were explanted and histological and SEM analyses were performed, as previously described.

RESULTS

1. AORTA ACELLULAR MATRICES

Two cycles of detergent-enzymatic treatment were needed to completely remove cells. Between the two decellularization cycles aortas were treated with Collagenase IV to detach the endothelial layer, still present at the end of the first treatment (Fig. 1 C). Collagenase digestion allowed us to decrease the treatment duration that would compromise the mechanical properties of the biomaterial. H/E staining confirmed the absence of cells (Fig. 1D). AAMs maintained elastic fibers, while connective component was modified compared to native tissue (Fig. 1A) by a longer detergent treatment (Fig. 1C).

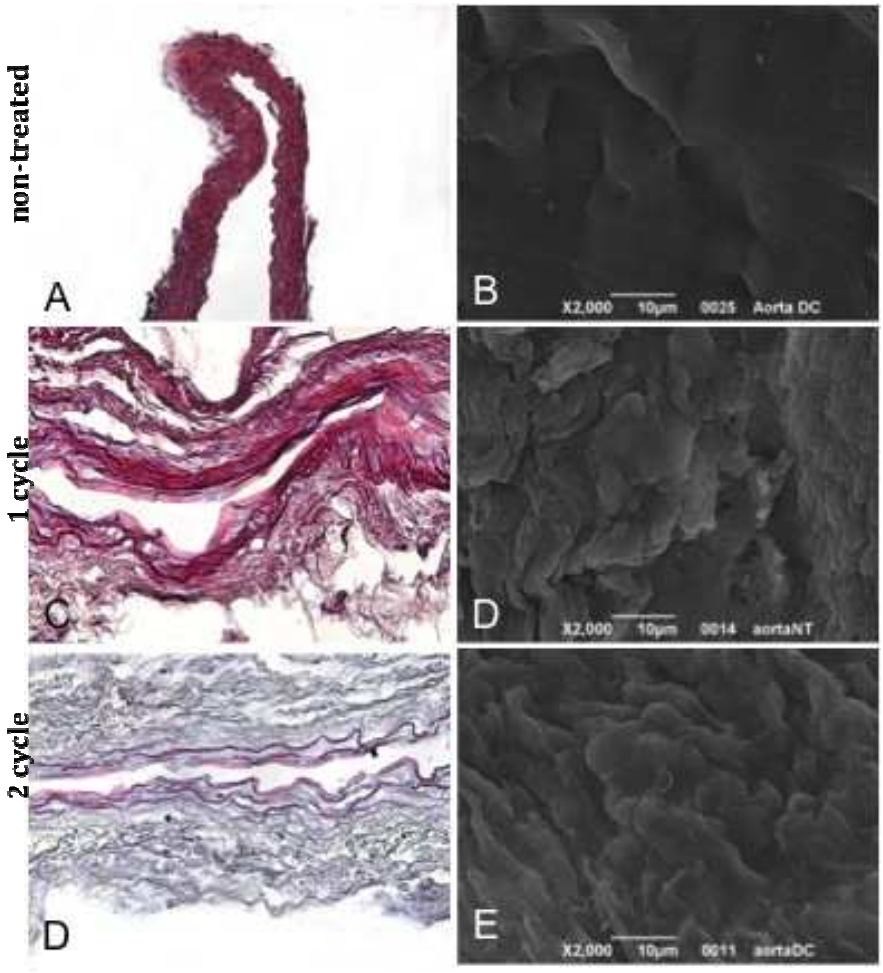


Figure 1 H/E staining (A,C,D; magnification x200) and SEM analysis (B, D, E; magnification x200) of not treated abdominal aorta (A,B), the same after one (C,D) or 2 (D,E) detergent-enzymatic treatments.

Immunohystochemistry revealed the absence in AAMs (Fig. 2 C, D) of both MHC I and II cell membrane antigens, normally present in native tissue (Fig. 2, A, B).

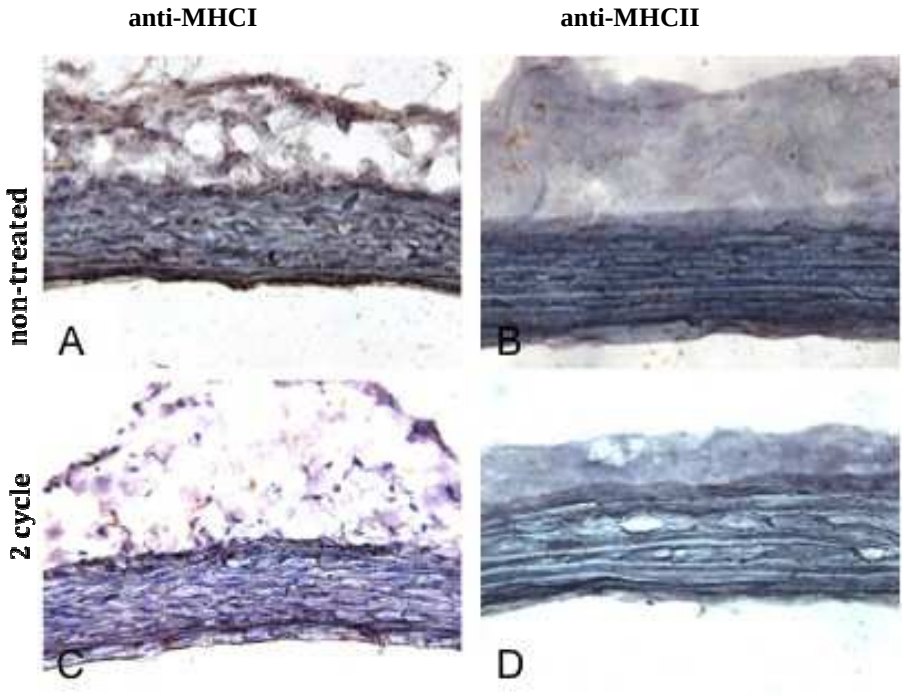


Figure 2 Immunohystochemistry performed using anti-MHC I (A,C) and II (B,D) antibodies before(A,B) and after 2 detergent-enzymatic treatments. Magnification x200.

2. CELL CULTURES

a- Skin microvascular endothelial cells: isolation and characterization

In 2 weeks, 6 millions of microvascular endothelial cells were obtained from a 2 cm^2 skin biopsy (Fig. 3 A, B). CD31 (Fig. 3C) and von Willebrand factor (Fig.3D) stainings confirmed the maintenance of the endothelial phenotype till the fourth culture passage.

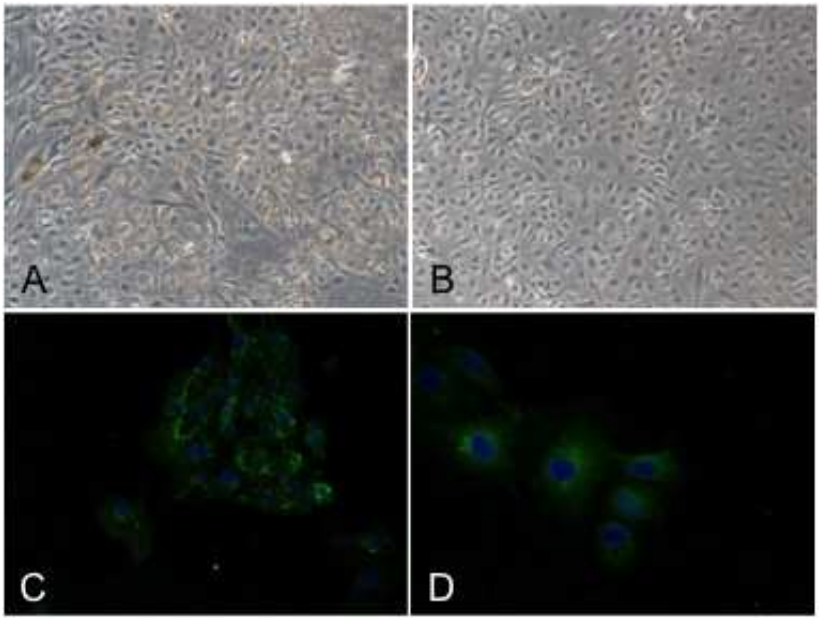


Figure 3 Cultures of skin microvascular endothelial cells. Phase-contrast microscopy (A,B, magnification x100). Immunohystochemistry performed using anti CD-31 (C) and anti von-Willebrand factor antibodies (D). Magnification x400

b- ECs/AAM cultures

ECs were seeded into the luminal surface of AAMs. At 72 h ECs adhered on AAMs, provided a continuous monolayer (Fig. 4 A, B, C) and maintained their phenotype (Fig. 4 D).

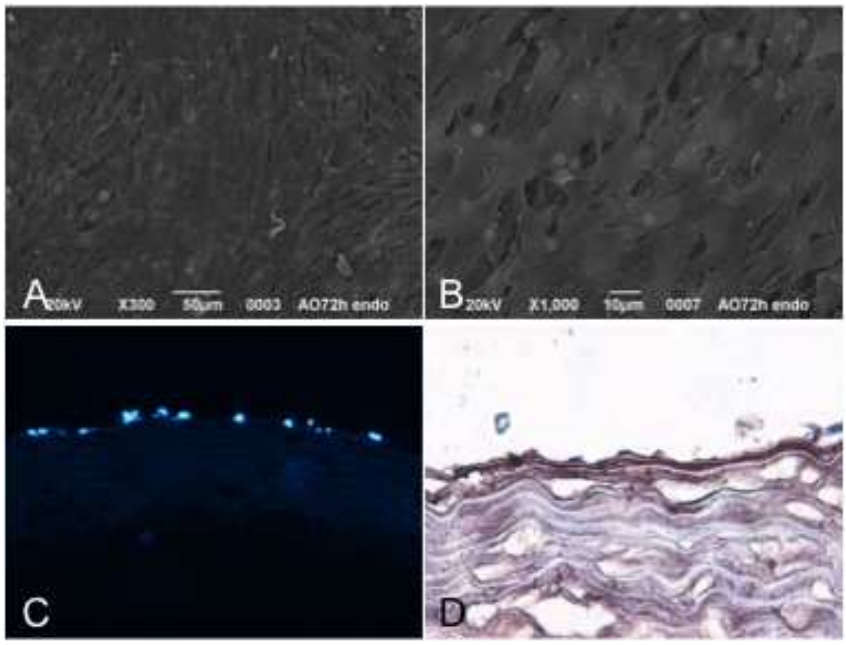


Figure 4 SEM (A, B; magnification x300, x1000) and DAPI staining (C, magnification x 200) of ECs/AAM cultures at 72h. The EC phenotype was confirmed by immunohystochemistry performed using an anti-von Willebrand factor antibody (D, magnification x400).

3*. IN VIVO* EXPERIMENTS

AAMs or AAMs plus cells were implanted into abdominal aorta of female Lewis rats. Animals have been sacrificed 1 or 3 months after surgery.

Group A: rats receiving only AAMs

Seven rats received only AAMs as aorta interposition graft: 4 animals were sacrificed after 1 month and 3 after 3 months. All animals survived.

Explanted grafts presented a higher diameter than host vessel (Fig. 5 A, B).

One month after surgery, only the borderline with the native vessel was reendothelized (Fig. 5 C), while the surface in the middle of the graft shown collagen fibers and platelets adhesion (Fig. 5 D).

Only one graft presented almost a continuous ECs monolayer (about 90% of surface area) and didn't present thrombi. However, histological analysis shown intimal

hyperplasia and a thick adventitial layer, while elastic fibers were well organized (Fig. 5 E).

Analogous results were obtained analysing samples collected three months after surgery. Two grafts still presented exposed collagen fibers remnants and ubiquitous platelet adhesion, while only one presented luminal surface almost completely reendothelizated. Moreover, histological analysis shown a severe intimal hyperplasia. However, no graft presented any thrombi.

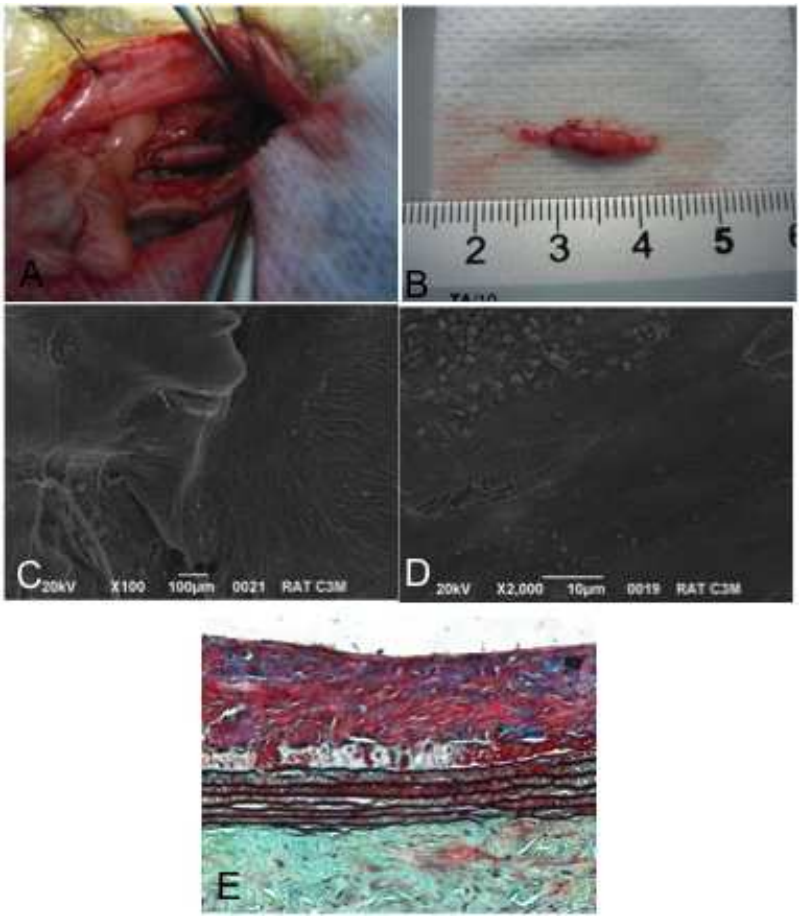


Figure 5 AAM grafts 1 month after surgery. Explanted graft (A,B); SEM of luminal surface (C,D); Movat staining (E, magnification x200)

Group B: rats receiving AAMs/ECs

Nine rats received AAM plus ECs as aorta interposition graft: 4 animals were sacrificed after 1 month, while 5 rats after 3 months. All animals survived and no thrombi were observed in all samples.

The diameter of the graft was the same as the host one (Fig. 6 A, B). All grafts were completely reendothelized with no platelet adhesion (Fig. 6 C, D). Although elastic

fibers were visible, the tunica media appeared thinner than the native one. As observed in AAM grafts, adventitial layer was hyperplastic.

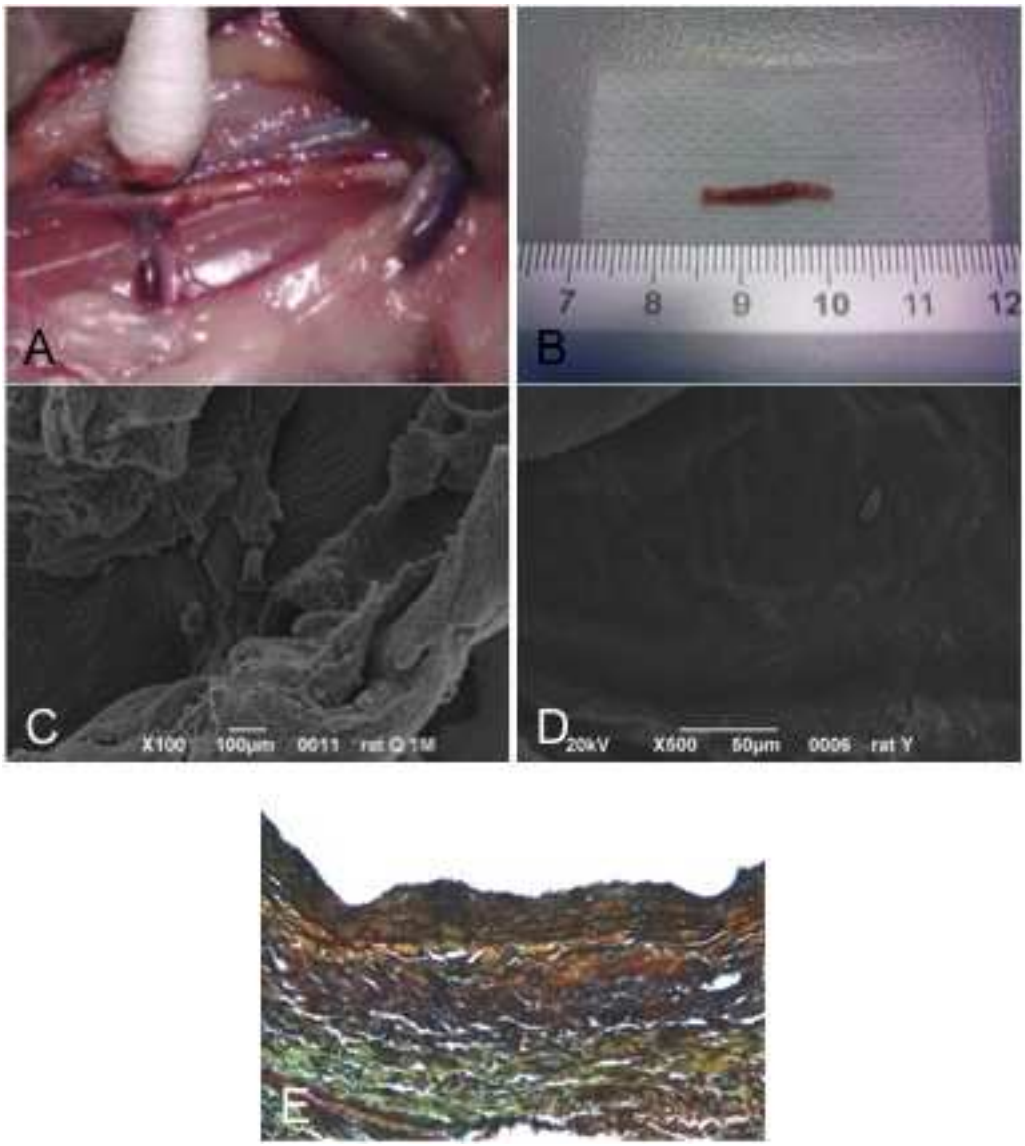


Figure 6 AAM plus ECs grafts 1 month after surgery. Explanted graft (A,B); SEM of luminal surface (A, B); Movat staining (F, magnification x200).

Three months after surgery grafts composed of AAM plus ECs appeared to be narrowed. Grafts were completely reendothelized with no platelet adhesion (Fig. 7 A, B). However, Movat staining shown a reduced elastic fiber layer (Fig. 7 C) comparated to the native one (Fig. 7 D), a moderate intimal hyperplasia and a tunica adventitia similar to the host one.

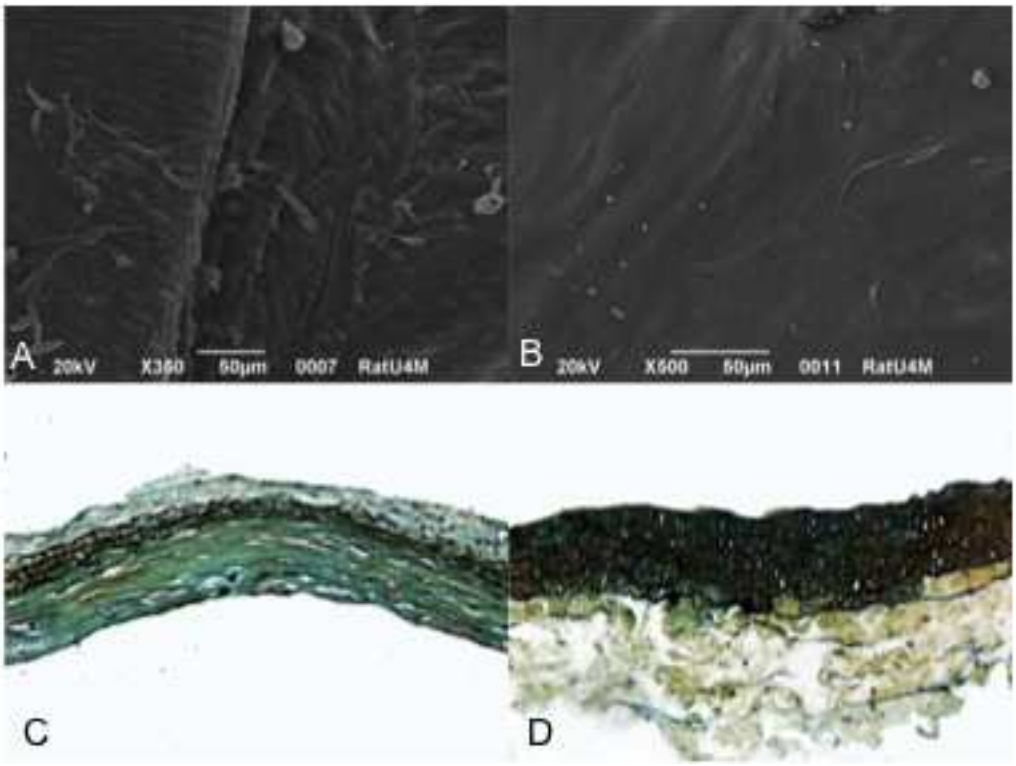


Figure 7 AAMs plus ECs grafts 3 months after surgery (A, B, C): SEM of luminal surface (A, B); Movat staining (C, magnification x200). Movat staining of native abdominal aorta (D).

DISCUSSION

This work has demonstrated that AAMs previously seeded with skin microvascular ECs could be used as vessel substitutes. AAMs were obtained following Meezan method with minor modifications. In previous studies, this immuno-enzymatic protocol has already shown to obtain suitable AMs that have been used both in preclinical and in clinical applications (Gilbert *et al*, 2006). This decellularization process induces the loss of the major histocompatibility complex markers, but maintains angiogenic factors, such as b-FGF and TGF-β (Ribatti *et al*, 2003; Conconi *et al*, 2004 *bis* and 2005 *bis*). Thus, AMs can present angiogenic activity that is an important factor for the *in vivo* integration of the tissue substitutes. AMs obtained by this protocol can support *in vitro* adhesion, growth and function of several cell types (Burra *et al* 2004; Dettin *et al* 2005; Conconi *et al* 2005), while *in vivo* AMs can act as a template allowing the ingrowth of the host cells and can be remodeled in a living tissue (Parnigotto PP *et al*, 2000; Marzaro M *et al*, 2006; Conconi MT *et al*, 2004). Moreover, they represent preformed structures whose length and gauges can be choice according to the dimension of the segment to be repaired. Another advantage is the possibility to have easy and unlimited availability of inexpensive grafts containing tissue-specific proteins. On June 2008 the first tissue engineered trachea, created using a AM human donor trachea as scaffold seeded with patient's own cells, was implanted into a 31-year-old woman's left main bronchus (Macchiarini *et al*, 2008).

In this work, AAMs maintained the three different layers as non-treated aorta and lacked of antigenic epitopes. Nowadays, several groups tested various AMs as vascular graft (Clarke et al, 2001; Conklin et al, 2002). Although they shown a good patency, the acellular luminal surface of these decellularized matrices without endothelial cells (ECs) lining carries a substantial risk for thrombosis when exposed directly to the blood flow. Our study confirmed these observations. Indeed, at 1 and 3 months, AAM grafts were not completely reendothelized, presented thrombi and intimal hyperplasia. Skin microvasculature could be an alternative source of ECs: skin biopsy is a moderately invasive procedure and in a short time allowed to obtain a large amount of cells from small samples. One month after implantation grafts composed by AAM and ECs could be histologically compared to native vessels, demonstrated a good patency and no thrombi. After three months, a moderate

intimal hyperplasia was detected and grafts presented the tunica media thinner than the one of native vessel. Although the substitutes appeared narrowed, patency was good and no thrombotic processes were visible. It can be supposed that the remodelling process was still in progress. Thus, longer endpoints should be investigated to determine the *in vivo* remodelling of the scaffold. However, these results agreed to the ones obtained by Kaushal *et al* (2001) seeding porcine iliac AMs with endothelial progenitors from peripheral blood. To improve the outcome of reconstructive surgery, alternative approaches should be evaluated. For example co-seeding of ECs and SMCs may be a suitable strategy to maintain the artery wall structure, but it will take a longer time to obtain the substitute (Neff *et al*, 2006).

In the present study we attempted to construct vessels in a clinically relevant time frame. Important to clinical application is having conduit available in a wide range of sizes on short notice. Despite other vascular regeneration techniques already used in clinical practise, which takes 4-6 weeks to create an implantable grafts (Neff *et al*, 2006), this protocol lead to obtain a vascular substitute in only 2 weeks. Besides this technique might eliminate the need to remove healthy vessels for collecting autologous endothelial cells.

CONCLUSION

Overall, this work has shown that the detergent-enzymatic treatment allows to obtain AMs possessing the main features of the native tissue. For this reason, tissue-specific AMs were able to act both as suitable environment for *in vitro* and *in vivo* growth and differentiation studies and as biomaterial to develop vessel substitutes.

In particular, KAMs supported *in vitro* both proliferation and differentiation of renal progenitors from amniotic fluid into tubular-like and podocyte-like cells. Furthermore, *in vivo* experiments shown that progenitors expressed mature renal markers, attracted inside KAMs differentiated murine cells and integrated into tubular host structures. These results could suggest to evaluate this approach for the enhancement of the function of hypoplastic kidneys.

On the other hand, AAMs seems to be an interesting biomaterial for vascular TE. The lack of reendothelization, leading to intimal hyperplasia and increased incidence of thrombosis observed in AAMs grafts, have indicated the need to provide *in vitro* an endothelial coverage of decellularized tissue. Indeed, grafts composed of AAM and skin microvasculature ECs shown good patency and no thrombi. Although these grafts appeared narrowed and a moderate hyperplasia has been detected in the inner layer, they presented two main advantages: they were obtained into a clinically relevant time frame and eliminated the need to remove healthy vessels for collecting autologous ECs.

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